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PRECIPITATION AND COMPLEMENT FIXATION REACTIONS WITH RESIDUE ANTIGENS IN THE NON-HEMOLYTIC STREPTOCOCCUS GROUP.

By CHARLES H. HITCHCOCK, M.D.

(From the Department of Internal Medicine of Washington University School of Medicine, St. Louis.)

(Received for publication, June 25, 1924.)

The orderly classification of non-hemolytic streptococci has met with the same difficulties as has that of the hemolytic group. Earlier studies failed entirely to recognize the distinction based upon the action of the organisms upon red cells, although this had been clearly pointed out as early as 1903. Later classifications based upon erythrocyte and fermentation reactions (1–3) were arbitrary, but were made to serve as a fair basis for the comparison of different strains. The agglutination and agglutinin absorption methods were found roughly to confirm them, but clear-cut results were not secured (4–7). The complement fixation reaction, as employed by Floyd and Wolbach (4), failed to yield additional information. Using the clear centrifugate from broth cultures, Barnes (8) studied flocculation reactions in immune sera, but failed to clarify the subject perceptibly. His results were later shown by Krumwiede and Valentine to have been in reality thread reactions (9).

Kinsella and Swift (10), employing antiformin antigens, showed by means of complement fixation that an inverse ratio exists between fixability of antigen and fixation range of corresponding antiserum, and were accordingly able to separate their organisms into two main groups, with a few connecting strains. Kinsella later (11) showed that one of these groups showed pronounced cross-fixation with the hemolytic family, whereas the other lacked this property entirely. Howell (12) was unable to confirm these results *in toto*, although she found a certain amount of irregular cross-fixation among her organisms.

In view of the striking neglect of the precipitin reaction, it seemed of interest to study by this method a few strains of non-hemolytic streptococci, with extracts similar to the "residue antigens" described by Zinsser and Parker (13) used as antigens. Similar or identical bacterial products have recently been shown by Avery and Heidelberger (14) to be type-specific within the pneumococcus family. As the application of this method to the non-hemolytic streptococci is more difficult than to the hemolytic family, it was found desirable in every instance to check the results by means of the reaction of complement fixation.

Methods.

1. Table I presents the twelve strains employed, with reference to their sources and properties. In every case the cultures were plated out at least twice upon blood agar, and each time single colonies were picked for isolation. Bile solubility tests were made by mixing 0.2 cc. of sterile ox bile with 0.8 cc. of 24 hour plain broth culture, and incubating in the water bath at 37° for 2 hours. For testing the effect upon red cells, 0.5 cc. of a 24 hour plain broth culture was mixed in a small tube with an equal volume of a 5 per cent suspension of washed sheep erythrocytes in saline, and the mixture was incubated in the water bath at 37° for 1 hour.

2. The preparation of the bacterial antigens has been previously described (15), and it is only necessary here to state that the method as outlined was rigidly adhered to.

3. Rabbits were used for the preparation of antisera. Immunization was effected by the intravenous injection of whole 18 hour cultures in plain meat infusion broth of pH 7.6, sterilized by heating in the water bath at 56° for 1 hour. The animals were at first given successive doses of 10 cc. each at 4 day intervals. After ten such injections, trial bleedings were made, and it was found that some of the sera were already rich in complement-fixing and precipitating antibodies. The animals yielding inactive or faintly active sera were given a further course of four injections at 4 day intervals, each dose consisting of the organisms sedimented by centrifugation from 50 cc. of plain broth culture and resuspended in 10 cc. of the supernatant liquid. After the lapse of 4 or 5 days, several of the animals were given a further similar course, each dose consisting of the organisms similarly recovered from 100 cc. of plain broth culture, and resuspended in 10 cc. of supernatant liquid. This additional dosage was occasionally successful, but showed in general little advantage over the smaller amounts. In addition, there was a tendency for weak sera to become entirely inactive under such intensive treatment. Long courses of 10 cc. doses at 4 day intervals were no more successful than the shorter ones. Finally, there was tried a method which had been found satisfactory for many pneumococci.

The animals were given on successive days three doses of 10 cc. each of the whole killed culture. After the lapse of a week followed three more doses, on 3 successive days, each dose consisting of the organisms from 50 cc. of plain broth culture, prepared as before noted. After the lapse of another week, trial bleedings were made, and such animals as were refractory were given a further course of three successive doses, each consisting of the organisms from 100 cc. of broth culture. If at the end of the ensuing week the serum of the animal was still inactive, it was found useless to proceed further. By one or another of these methods, it was found possible to prepare antisera to most of the strains.

4. The technique of the precipitation tests corresponded with that previously described (15), with the exception that in each case 0.1 cc. of the immune serum was used in place of 0.2 cc.

TABLE I.

Strains of Streptococci Employed in This Study.

Source.	Erythrocytes.	Bile.
A4, blood culture; bacterial endocarditis.	Methemoglobin.	Insoluble.
A49, " " rheumatic fever.	"	"
A135, " " " "	"	"
A179, heart valve (necropsy); rheumatic endocarditis.	"	"
2B, blood culture; bacterial endocarditis.	"	"
B3, normal throat.	"	"
B4, " "	"	"
2C, sputum; bronchial asthma.	"	"
C3, " bronchopneumonia.	"	"
2D, blood culture; bacterial endocarditis.	"	"
38D, " " rheumatic fever.	"	"
MB, " " bacterial endocarditis.	Indifferent.	"

5. The complement fixation reactions were performed with the following quantities. 0.1 cc. of bacterial extract was used as antigen. The system was the usual rabbit anti-sheep combination, two units each of complement and of amboceptor being employed. Five descending dilutions of immune serum were used; *viz.*, 0.1 cc., 0.5 cc., 0.25 cc., etc. The antigen-serum-complement mixtures were diluted to 1.5 cc. with saline, and were incubated in the water bath at 37° for 1 hour. At the end of this time, sensitized cells prepared by mixing equal parts of washed 5 per cent sheep cells in saline and of diluted amboceptor were added in amounts of 1 cc. and the mixture was incubated in the water bath at 37° for 45 minutes. At the end of this time the tubes were promptly removed to the ice box, and were allowed to remain there overnight before final reading.

TABLE II.

Complement Fixations with Residue Antigens.

Antigen.	Serum.											
	2B	A49	MB	A179	A135	C3	38D	2C	2D	A4	B3	B4
2B	4	4	2	0	0	0	0	0	0	0	0	0
	4	4	3	0	0	0	0	0	0	0	0	0
	4	4	3	0	0	0	0	0	0	0	0	0
A49	0	4	4	3	3	0	0	0	0	0	0	0
	0	4	4	4	1	0	0	0	0	0	0	0
	0	4	4	4	0	0	0	0	0	0	0	0
MB	0	0	4	4	3	4	4	0	0	0	0	0
	0	0	4	4	3	4	4	0	0	0	0	0
	0	0	4	4	0	4	4	0	0	0	0	0
A179	0	0	4	4	0	4	0	0	0	0	0	0
	0	0	4	4	0	4	0	0	0	0	0	0
	0	0	4	4	0	4	0	0	0	0	0	0
A135	0	0	3	0	4	1	0	0	0	0	0	0
	0	0	4	0	4	0	0	0	0	0	0	0
	0	0	4	0	4	0	0	0	0	0	0	0
C3	0	0	4	4	0	4	4	0	0	0	0	0
	0	0	4	4	0	4	1	0	0	0	0	0
	0	0	4	4	0	4	0	0	0	0	0	0
38D	0	0	0	0	0	1	4	0	0	0	0	0
	0	0	0	0	0	1	4	0	0	0	0	0
	0	0	0	0	0	0	4	0	0	0	0	0
2C	0	0	0	0	0	0	4	0	3	0	0	0
	0	0	0	0	0	0	3	0	4	0	0	0
	0	0	0	0	0	0	1	0	4	0	0	0
2D	0	0	0	0	0	0	2	0	3	0	0	0
	0	0	0	0	0	0	2	0	4	0	0	0
	0	0	0	0	0	0	0	0	3	0	0	0
A4	0	0	0	0	0	0	0	0	0	4	0	0
	0	0	0	0	0	0	0	0	0	4	0	0
	0	0	0	0	0	0	0	0	0	4	0	0

4 indicates complete lack of hemolysis; 3, 25 per cent of cells hemolyzed; 2, 50 per cent of cells hemolyzed; 1, 75 per cent of cells hemolyzed; 0, complete hemolysis.

TABLE II—*Concluded.*

Antigen.	Serum.											
	2B	A49	MB	A179	A135	C3	38D	2C	2D	A4	B3	B4
B3	0	0	0	0	0	0	0	0	0	0	4	4
	0	0	0	0	0	0	0	0	0	0	4	4
	0	0	0	0	0	0	0	0	0	0	1	3
B4	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0

TABLE III.

Precipitation Reactions with Residue Antigens.

Antigen.	Serum.											
	2B	A49	MB	A179	A135	C3	38D	2C	2D	A4	B3	B4
2B	++	0	0	0	0	0	0	0	0	0	0	0
A49	0	+	+	+++	+	0	0	0	0	0	0	0
MB	0	0	++	++++	+++	+++	0	0	0	0	0	0
A179	0	0	+	++++	0	++++	0	0	0	0	0	0
A135	0	0	+	0	++++	0	0	0	0	0	0	0
C3	0	0	+	++++	0	++++	0	0	0	0	0	0
38D	0	0	0	0	0	0	±	0	0	0	0	0
2C	0	0	0	0	0	0	++	0	+++	0	0	0
2D	0	0	0	0	0	0	++	0	++	0	0	0
A4	0	0	0	0	0	0	0	0	0	+++	0	0
B3	0	0	0	0	0	0	0	0	0	0	++	++
B4	0	0	0	0	0	0	0	0	0	0	0	0

++++ indicates thick, matted precipitate, not broken up even upon violent agitation; +++ indicates same, but less in amount; ++, coarse, flocculent precipitate in bottom of tube; +, definite clouding of liquid, but with little or no settling of the precipitate to the bottom of the tube; ±, faint clouding of liquid, not constantly present in repeated observations; 0, neither clouding nor precipitation.

RESULTS.

These are presented in summary in Tables II and III.

In every instance, a series of controls was made, with normal rabbit serum substituted for the immune serum. In addition, each

of the immune sera was tested by both methods with four antigens prepared as usual from each of four strains of hemolytic streptococci, and also with antigens prepared from a series of pneumococci, including one strain each of Type I, Type II, Type II atypical, and Type III, and ten strains of Type IV. That each of these control antigens was efficient was shown by the production of heavy precipitates when mixed with the corresponding immune horse or rabbit serum. In no case was cross-precipitation or cross-complement fixation noted between non-hemolytic streptococcus sera and either hemolytic streptococcus or pneumococcus antigens. That non-hemolytic antigens will not give cross-precipitation with hemolytic antisera has already been shown (15) and other studies[1] have demonstrated that they are likewise inert when mixed with antipneumococcus sera. The chain of evidence is thus completed, that, as far as concerns these residue antigens, the hemolytic streptococci, the non-hemolytic streptococci, and the pneumococci form antigenically distinct groups.

The entire absence of non-specific flocculations with normal rabbit serum must likewise be emphasized, as contributing further evidence to the specificity of these bacterial extracts.

DISCUSSION.

A glance at the tables shows that the uniformity noted in the hemolytic family is strikingly absent. Some strains are apparently quite isolated individuals; others show a certain amount of cross-reaction between them, but this is not orderly enough to permit it to be used as a basis for classification. It is, however, possible that the study of a larger collection of organisms would display general tendencies which are missing when the number is restricted. No grouping according to source or disease process is evident. Neither is there evidence of the inverse relationship between fixability of antigen and range of antiserum which has been demonstrated by the use of a different technique. The conclusion cannot be avoided that the latter phenomenon, as well as that of cross-reaction between hemolytic and non-hemolytic groups, is not associated with the precipitable substance extracted by the method described. Such cross-reactions

[1] Unpublished data.

may be conceived as being properties of the protein or lipoid-protein fraction of the bacterial cell; or it is possible that the more concentrated antiformin solutions used by Kinsella are able to split the molecule of the precipitable substance into simpler compounds with wider reactive tendencies. Experiments to decide this point are at present under way.

SUMMARY AND CONCLUSIONS.

The applicability to the non-hemolytic streptococci of the technique used for the extraction of a soluble precipitating substance from the hemolytic streptococci is demonstrated.

With this substance as antigen, it is demonstrated by means of precipitation and complement fixation that the non-hemolytic streptococci form an antigenically distinct, but entirely heterogeneous group.

Such cross-reactions as do occur within this group are not useful in the systematic classification of these organisms.

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

VI. Variations in Growth and Malignancy of Transplanted Tumors.

Part 1. Results of Transplantation for the First Twenty Generations.

By WADE H. BROWN, M.D., LOUISE PEARCE, M.D., and CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 23, 1924.)

In previous papers of this series (1–5) we have reported the results of our study of a spontaneous tumor of the rabbit and of various methods of transplantation during the earlier generations, in so far as they pertain to the essential features of the growth of primary tumors, the course of the disease, and the occurrence and distribution of metastases.

It has been pointed out that the results of transplantation varied greatly, depending on the method employed, and that, while with some methods it was difficult to obtain even a primary growth, other methods gave an almost uniform series of takes, and that intratesticular inoculation produced a growth that was capable of metastasizing to almost all parts of the body. It was noted, however, that the tumors of individual animals of a given series varied from a small local growth of benign character to tumors that were sufficiently malignant to cause the death of the animal within a few weeks after inoculation. Reference was also made to the fact that similar variations in malignancy were shown by different series of animals and that in addition to such factors as the method of inoculation and inequalities in the resistance of individual animals, the malignancy of the tumor appeared to be influenced to some extent by such factors as continued passage, the age and the breed of the animal, the character of the material used, and the time at which the inoculations were made (season of the year). In other words, it appeared that the growth and malignancy of the

tumor might be affected intentionally or accidentally by numerous conditions and that before any attempt could be made to plan experiments, or to evaluate the results of experimental procedures whose object was to increase or diminish the malignancy of the tumor, it would be necessary to study the effects of those influences that were most likely to play a part in experiments of this kind.

A simple method of approaching this problem was afforded by an analysis of the results obtained in the course of routine transplantation, and for this purpose we have used material available from the first twenty generations of intratesticular transplants. The present paper serves a double purpose, therefore, in that it contains a report of the results of transplantation, as well as a discussion of unintentional and to some extent uncontrollable sources of variation in the growth and malignancy of the tumor. Part 1 of this paper will be devoted to a systematic presentation of the results of transplantation for the first twenty generations.[1] These results will be discussed in the second section of the paper from the standpoint of the influence of such factors as passage, the material used for inoculation, the age and the breed of the animal, and the time, or season of the year, at which the inoculations were made. Preliminary reports dealing with certain aspects of the subject of the growth and malignancy of the tumor have already appeared (6–10).

EXPERIMENTAL.

This report is based on the results obtained in 237 rabbits of the first twenty generations inoculated between March 10, 1921, and December 13, 1922, as recorded briefly in Text-figs. 1 to 4.

Experimental Conditions.—With few exceptions, the animals received a single unilateral testicular inoculation of a cell emulsion prepared by methods described in previous papers. The dose used varied from 0.2 to 0.3 cc. The rabbits in Group B of Generation XVI were inoculated in both testicles, with an interval of 10 days between the two inoculations. There were, also, two groups of animals that were inoculated with an emulsion that had been subjected to freezing and thawing, and grinding in the frozen state (Nos. VII B and VIII C).

Other irregularities in the experimental conditions that should be noted concern the question of castration before or after inoculation and the length of the period of observation. In a few instances, the testicle containing the tumor

[1] The tumor is now (May, 1924) in its thirty-third generation.

was removed soon after inoculation, but the general results appear to have been disturbed very little by such conditions. On the other hand, the removal of one testicle a week before the inoculation of Rabbits 11 to 15 inclusive of Generation VIII was associated with the occurrence of two cases of extreme malignancy.

During the first ten generations the observation period was very irregular. Some animals were held as long as 4 to 7 months, while others were killed as early as 2 to 3 weeks after inoculation. This was due, in part, to the relatively slow progress of the disease during the early generations and, in part, to an attempt to follow the course of events by postmortem examinations. As the work progressed these conditions were equalized to some extent and an effort was made to fix the period of observation according to the progress of the disease so as to give a time value to the results.

Method of Charting Results.—The experimental conditions and the results obtained in each series of animals are recorded graphically in Text-figs. 1 to 4 in the order of generations, which does not conform to the time order of inoculation. Three major divisions have been made on these charts. The first section (left) gives experimental data including the generation of the transfer, together with subdivisions of the generation when different emulsions were used, the date of the inoculation, and the age group and type of the animal. The second section of the charts contains the results of clinical observations and is divided into two parts. The first of these gives data pertaining to the primary tumor, while the second contains observations on the occurrence of metastases that could be recognized clinically.

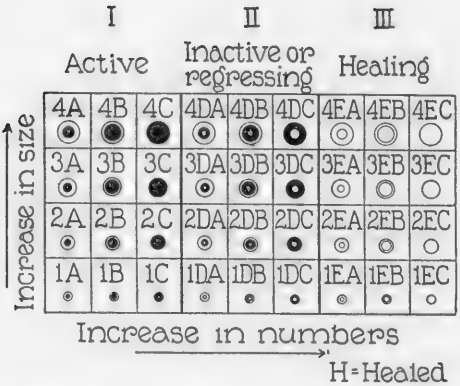
The data in the first two sections of the charts are not as complete as might be desired owing to the fact that it was not possible to maintain detailed records of the clinical course of events in all cases, and in some instances in which the available data were not sufficiently definite it seemed best to omit them altogether.

The greater part of the data recorded in Text-figs. 1 to 4 refers to results of postmortem examinations. In the first column of this section we have noted any decided abnormality in the physical condition of the animal at the time of death or at the time the animal was killed, in so far as such information was available. The next two columns indicate what eventually happened to each animal. It will be noted that comparatively few deaths are recorded, due to the fact that in order to avoid loss from postmortem decomposition, animals were usually killed when it became apparent that death was imminent. But, from the data given, the probable fatalities and the approximate length of survival can be estimated in nearly all cases. The considerations that governed the time of killing other animals have already been mentioned.

The remaining sections of Text-figs. 1 to 4 record the state or fate of the primary tumor, or of a recurrent growth in instances in which the primary tumor had been excised, as regards size, the relative proportions of living and necrotic tissue, the degree of encapsulation, and the progress of healing, together with

Explanation of Text-Figs. 1 to 4.

Rate of growth		Encapsulation	
Slow	<	Thin	—
Moderate	<	Slightly thick	—
Active	<	Moderately thick	—
Rapid	<	Heavy	—



The system of symbols used in Text-figs. 1 to 4 to describe primary and secondary tumors.

Age Groups.—I indicates young; II, young adults; III, adults; IV, old.

Types.—A. indicates albino; Bd., brindle; Bg., Belgian; Bl., black; Br., brown; Bt., Dutch belt; F., Flemish; Fn., fawn; G., gray; Hm., Himalaya; M., Maltese; N.Z., New Zealand; T., tan; W., white (in color combination).

Terminal Condition.—C. indicates cachectic; M., moribund; P., paralysis; Pr., poor condition; Th., thin; W., weak.

Disposition.—D. indicates dead; Dis., discarded; K., killed; R., reinoculated.

Text-figs. 1 to 4 show the results of tumor growth for the first twenty generations.

CLINICAL

POSTMO

	Generation	Series	Date of inoculation	Animal number	Age group	Type	Rate of growth	Maximum size	Time - weeks	Cord extension	Edema	Castrated	Recurrence	R eyes	L eyes	Skin	Muscles	Bones	Subcutaneous tissues	Superficial lymph nodes: Inguinal-flank	Superficial lymph nodes: Axillary-mammary	Superficial lymph nodes: Cervical-submental	Uninoculated testicle	Condition	Termination	Time - weeks	Primary or recurrent tumor: State of growth	Encapsulation	R cords
	Primary tumor													Metastases (Days after inoculation)															
1920	Spontaneous tumor	10-1			IV	A	^^^	⊙	2		Excised	18	12-28							96				M	K	23			
							^^^	⊙	=		"	16	1-24																	
							●		6																					
1921	I		3-10	1			<	●	10	●	88														K	14	◔	▬	●
				2				±?																	=	=	–		
				3			<	●	=	⊙		57		196				196							=	26	◔	▬	⊙
				4			<	⊙	9																=	=	◔		
	II		4-7	1	II	T	<	⊙	8															Pr	K	24	⊙	▬	
				2	"	GW	^	●	9	●														Wk	=	10	◔		●
				3	III	A	^	●	4			33	–												=	9	Castrated		
				4	II	Br	^	●	=	⊙		=	+												=	8	⊙		⊙
				5	"	"	^	●	9																D	11	◔		In
				6	"	F	^	●	8			49	+												K	24	⊙	▬	
				7	"	G	^	●	7	⊙															=	7	◔	–	⊙
				8	"	"	^	●	4			33	+												=	9	⊙		
				9	III	Br	^	●	9	●														C	=	29	◔	▬	●
				10	"	"	^	●	4																=	4	◕	–	
	III	A	5-10	1	II	Br	^	●	3		21														K	33	H		
				2	"	A	?	±?	1-																D	1-	?		In
				3	III	G	<	⊙	2+															Wk	=	19	H		
				4	"	A	^	●	4															M	K	17			
				5	II	Br	^	●	2+		16														=	33	H		
				6	III	B1	<	⊙	4	●							133							Wk	D	23	◔	▬	⊙
		B	5-26	1	"	Bg	<	●																	=	11	◔	▬	●
				2	"	A	<	⊙																	R	16	H		
				3	"	"	<	⊙																	=	=	"		
				4	II	G	<	●	8	●														C	D	8+	◔	▬	⊙
				5	III	Br	^	●	4	⊙		28	–												K	8	Castr.		⊙
	IV		6-23	1																					D	6+	⊙		
				2								62	–												=	9	Castrated		
				3																					K	16	○	▬	
				4																					=	6	◔		⊙
				5																					=	16	H		
				6																					=	=	○	▬	
				7																					D	6+	◔		
				8																					K	16	H		
				9																					D	12	±?		
				10																					K	16	○	▬	
	V		8-24	1			<	⊙	6	●															K	8	H		⊙
				2			<	●	8	⊙	44														=	=	◔	▬	⊙

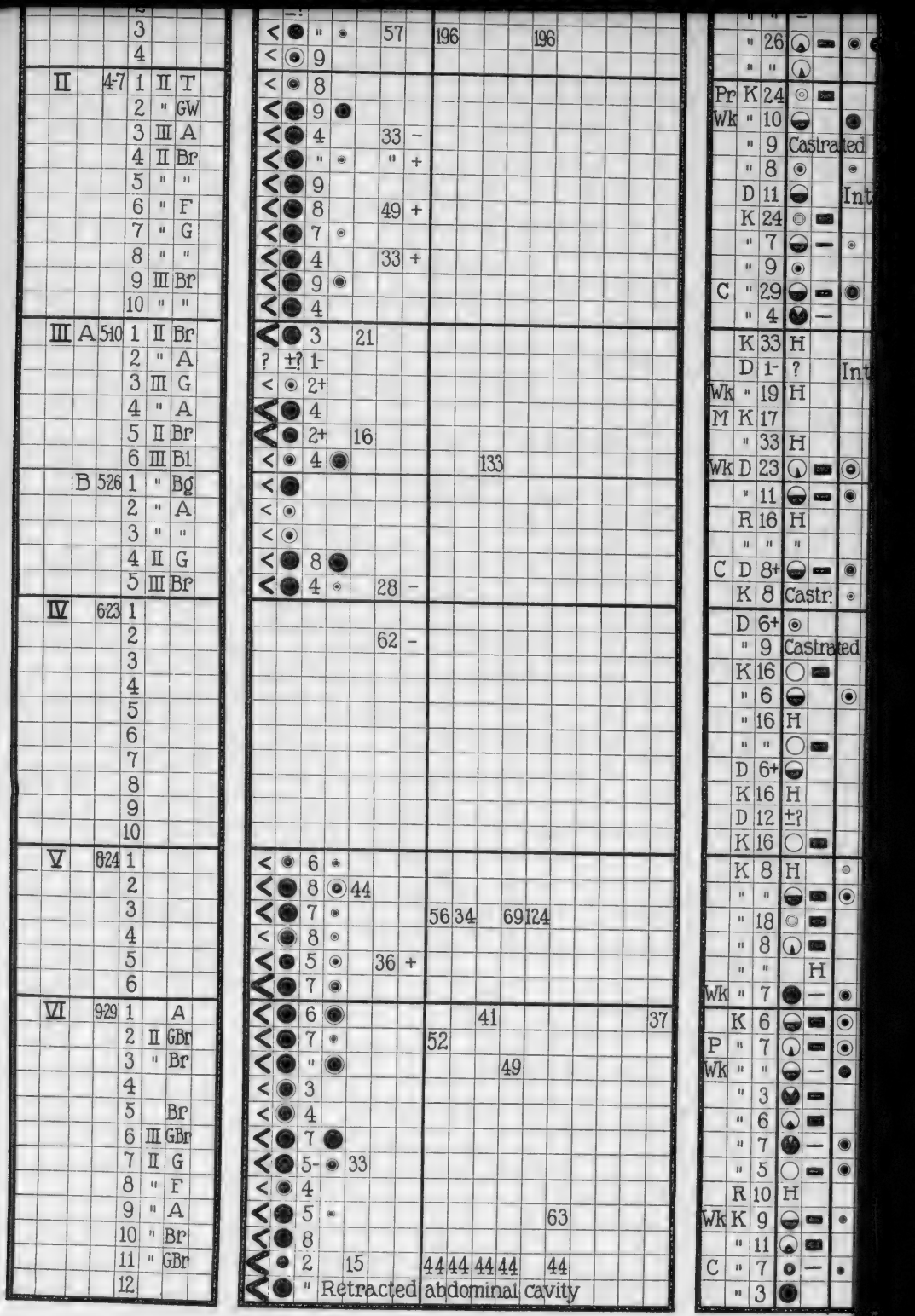
OSTMORTEM

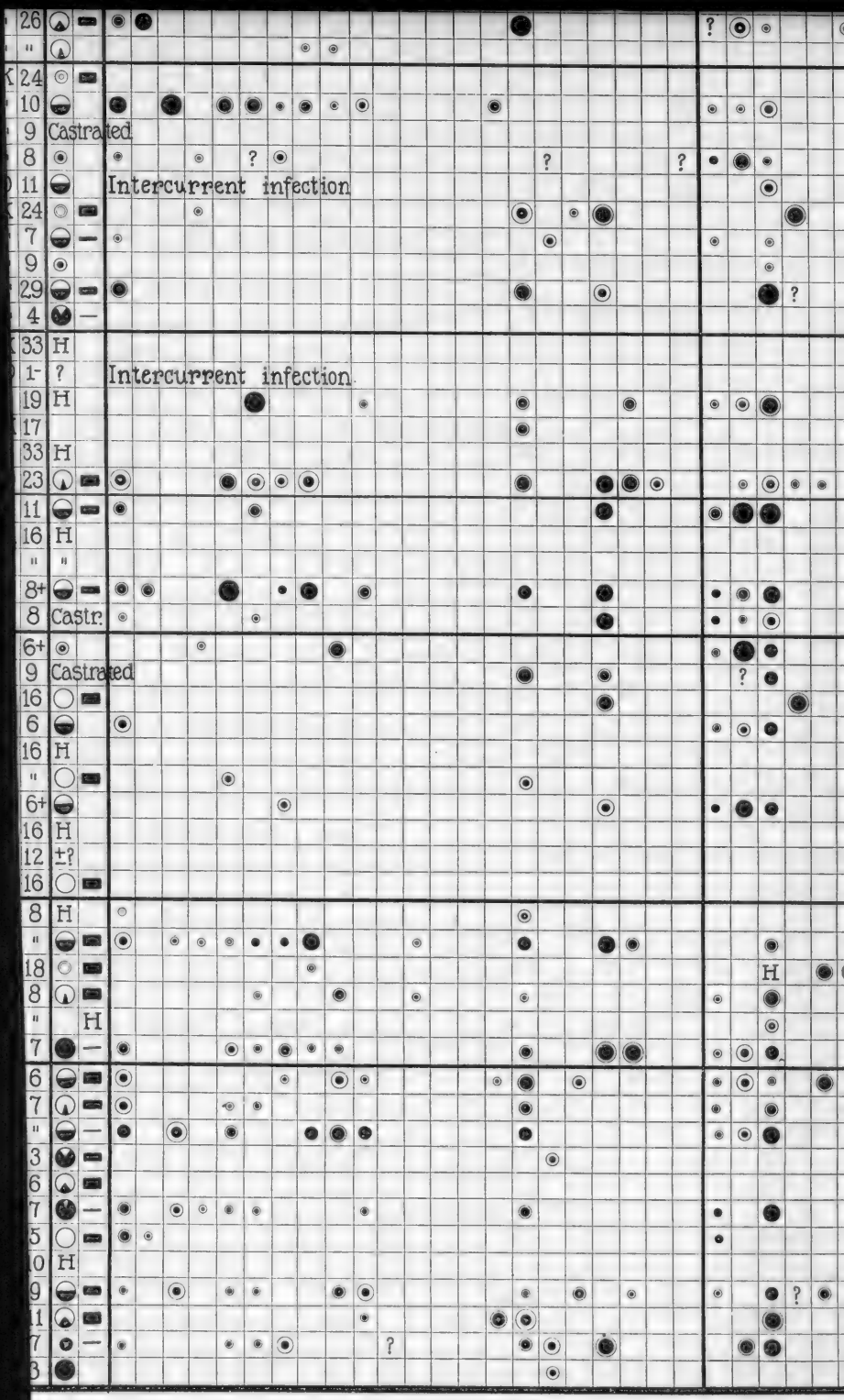
Termination Time-weeks	Primary or recurrent tumor: State of growth	Primary or recurrent tumor: Encapsulation	Extensions and implantations: R } cords L }	Tunics	Scrotum	Omentum	Mesentery	Diaphragm	Parietal peritoneum	Abdominal fat	Intestines	Stomach	Pancreas	Bladder	Seminal vesicles	Abdominal walls	Retroperitoneal tissues	Deep lymph nodes: " & mesenteric }	Peritracheal-peribronchial }	Mediastinum	Parietal pleura	Pericardium	Epicardium	Lungs	Liver	Kidneys
K 23									◉									●						●	●	⊙
K 14	◒	▬	●	●																⊙						⊙
"	–																									
26	◔	▬	⊙	●													●							?	◎	⊙
"	◔								⊙	⊙																
K 24	○	▬																								
10	◒		●		●		◉	◉	⊙	◉	⊙	⊙				●								⊙	⊙	⊙
9	Castrated																									
8	⊙		⊙			⊙		?	⊙									?					?	●	◉	⊙
11	◒		Intercurrent infection																							◉
K 24	○	▬				⊙											◉		⊙	◉						
7	◒	—	⊙															⊙						⊙		⊙
9	⊙																									⊙
29	◒	▬	●														●			⊙						◉
4	◕	—																								
K 33	H																									
D 1-	?		Intercurrent infection																							
19	H						◉				⊙						◉				⊙			⊙	◉	◉
K 17																	◉									
33	H																									
D 23	◔	▬	◎			●	◉	⊙	◉								●			●	◉	⊙			◎	⊙
11	◒	▬	◉				◉													●				◉	●	●
R 16	H																									
"	"																									
D 8+	◒	▬	◉	◉		●		•	◉		◉						●			●				•	◉	●
K 8	Castr.		⊙				⊙													●				•	⊙	◎
D 6+	⊙				⊙					◉														⊙	●	⊙
9	Castrated																●			⊙					?	●
K 16	○	▬																		●						
6	◒		◉																					◉	⊙	●
16	H																									
"	○	▬				⊙											⊙									
D 6+	◒							⊙												⊙				•	●	●
K 16	H																									
D 12	+?																									
K 16	○	▬																								
K 8	H		◉														◉									

Distant metastases

R } suprarenals
L }
R } eyes
L }
Hypophysis
Pineal
Thyroid
Parathyroids
Cranial cavity
Spinal canal
Uninoculated testicle
Spleen
Thymus
Inguinal-flank
Axillary-mammary
Cervical-submaxillary
Facial
Calvarial
Spinal
Thoracic
Anterior extremities
Posterior "
" "
Anterior "
Shoulder & thoracic
Cervical
Facial
Abdominal walls
Lumbar
Heart
Skin
Subcutaneous tissues

Superficial lymph nodes
Bones
Muscles


II 4-7 1 II T
2 " GW
3 III A
4 II Br
5 " "
6 " F
7 " G
8 " "
9 III Br
10 " "
III A 510 1 II Br
2 " A
3 III G
4 " A
5 II Br
6 III Bl
B 526 1 " Bg
2 " A
3 " "
4 II G
5 III Br
IV 623 1
2
3
4
5
6
7
8
9
10
V 824 1
2
3
4
5
6
VI 929 1 A
2 II GBr
3 " Br
4
5 Br
6 III GBr
7 II G
8 " F
9 " A
10 " Br
11 " GBr
12
57
196 196
33 -
" +
49 +
33 +
21
16
133
28 -
62 -
44
56 34 69 124
36 +
41 37
52
49
63
15
44 44 44 44 44
Retracted abdominal cavity
26
Pr K 24
Wk " 10
" 9 Castrated
" 8
D 11 Int
K 24
" 7
" 9
C " 29
" 4
K 33 H
D 1- ? Int
Wk " 19 H
M K 17
" 33 H
Wk D 23
" 11
R 16 H
" " "
C D 8+
K 8 Castr.
D 6+
" 9 Castrated
K 16
" 6
" 16 H
" "
D 6+
K 16 H
D 12 ±?
K 16
K 8 H
" "
" 18
" 8
" " H
Wk " 7
K 6
P " 7
Wk " "
" 3
" 6
" 7
" 5
R 10 H
Wk K 9
" 11
C " 7
" 3



TEXT-FIG. 1. Tumor growth. Generations I to VI.

CLINICAL

POSTMORT

Generation	Series	Date of inoculation	Animal number	Age group	Type
VII	A	10-8	1	I	Bg
			2		
			3	II	G
			4	III	MW
			5	II	A
			6	III	GBr
	B	Freezing & thawing	1	"	Br
			2	II	"
			3	III	Bl
			4	II	GBr
			5		
VIII	A	11-1	1	II	G
			2	III	A
			3	II	G
			4	"	A
			5		Fn
			6	IV	F
			7	III	G
			8	II	A
			9	III	"
			10	"	"
		Old Castrated, uninoc T.	11	"	Br
			12	II	GBr
			13	III	A
			14	"	G
			15	"	M
			16	IV	NZ
			17	"	F
			18	"	"
		Young	19	I	NZ
			20	"	F
			21	"	A
			22	"	Bg
			23	"	Br
			24	II	F
			25	"	Bl
			26	III	A
	B	11-9	1	"	"
			2	"	M

Primary tumor

Rate of growth	Maximum size	Time - weeks	Cord extension	Edema	Castrated	Recurrence
^	●	2				
^	⊙	1				
^^	●	3	•			
^	●	4				
^	●	3				
<	⊙	2				
^	●	6	◉			
	±?	3				
^^	●	10	◉			
^	●	9				
	±?	1				
?	Retracted abdominal cavity					
"						
"		"				
^	●	5				
^	●	=	⊙			
^	●	4				
^	●	5				
^	●	4	●	25		
^	●	=	⊙			
^	●	7	●			
?	±?	Intercurrent infection				
^	●	5	●			
^	●	7	●			
^	●	5	●			
^	●	4	◉			
?	±					
^	●	9	●			
^	●	6	◉			
^	●	2		14		
^	●	6				
^	●	2		14		
<	⊙	4	?			
^	●	=	◉			
^	●	3				
^	●	=				
^	●	5				
<	⊙	2				
?	±	Intercurrent infect				

Metastases (Days after inoculation)

R eyes	L eyes	Skin	Muscles	Bones	Subcutaneous tissues	Superficial lymph nodes: Inguinal-flank	Axillary-mammary	Cervical-submental	Uninoculated testicle

OSTMORTEM

Termination	Time-weeks	Primary or recurrent tumor: State of growth	Primary or recurrent tumor: Encapsulation	Extensions and implantations: R cords	L cords	Tunics	Scrotum	Omentum	Mesentery	Diaphragm	Parietal peritoneum	Abdominal fat	Intestines	Stomach	Pancreas	Bladder	Seminal vesicles	Abdominal walls	Retroperitoneal tissues & mesenteric	"	Peritracheal-peribronchial (Deep lymph nodes)	Mediastinum	Parietal pleura	Pericardium	Epicardium	Lungs	Liver	Kidneys	R suprarenals	L
X	2	●	▬																											
"	"	◉	▬																											
"	3	●	▬	●		◉													●											
"	10	◑	▬																											
"	7	◎	▬	◉																										
R	"	H																									◎			
X	8	○	▬	◉																										
"	3	±?																									◉			
"	12	◕	▬	◉	◎				◉				◉									◉								
"	11	◑	▬																											
"	1	±?																												
X	16	H																												
"	1	?		Intercurrent infection																										
"	10	◑	▬																							?				
"	8	○	▬																											
"	16	H		◎			◎	◎	◎		◎								●							●		◎	◉		
"	"	"																												
"	"	"																												
"	"	◑	▬	●	●		◎		◎							◎											◎			
"	14	◑	▬																●			◉				?				
"	10	◐	▬	●	◉	●		◎		◎	●	●																		
"	18	±?																												
"	7	◕	—	●	●		◎	●	●		●	●	●			◎				●		●					●	◉	◉		
"	"	◑	—	●	◉	●	◎					●	●				◎		◉	●	◎	●					●	◉	◉		
"	16	H						◎																				◎			
"	"	"										H																H			
"	18	–																												
"	"	◑	▬	◎					◎																					
"	14	◑	▬	◎			◎												◉			◉			◎	●			◉	
"	16	H																												
"	14	◑	▬																◉			◉								
"	15	H																										?		
"	"	"		◎																										
"	23	"																												
"	3	◉																												
"	"	◑		◎															◉											
"	8	○	▬																							●				
X	2	◉								?																?				
"	16	±?																										◉		
"	3	◉		◉																										

Distant metastases

R } eyes
L }
Hypophysis
Pineal
Thyroid
Parathyroids
Cranial cavity
Spinal canal
Uninoculated testicle
Spleen
Thymus
Inguinal-flank
Axillary-mammary
Cervical-submaxillary
Facial
Calvarial
Spinal
Thoracic
Anterior extremities
Posterior "
" "
Anterior "
Shoulder & thoracic
Cervical
Facial
Abdominal walls
Lumbar
Heart
Skin
Subcutaneous tissues

Superficial lymph nodes
Bones
Muscles

H

? ? ? "

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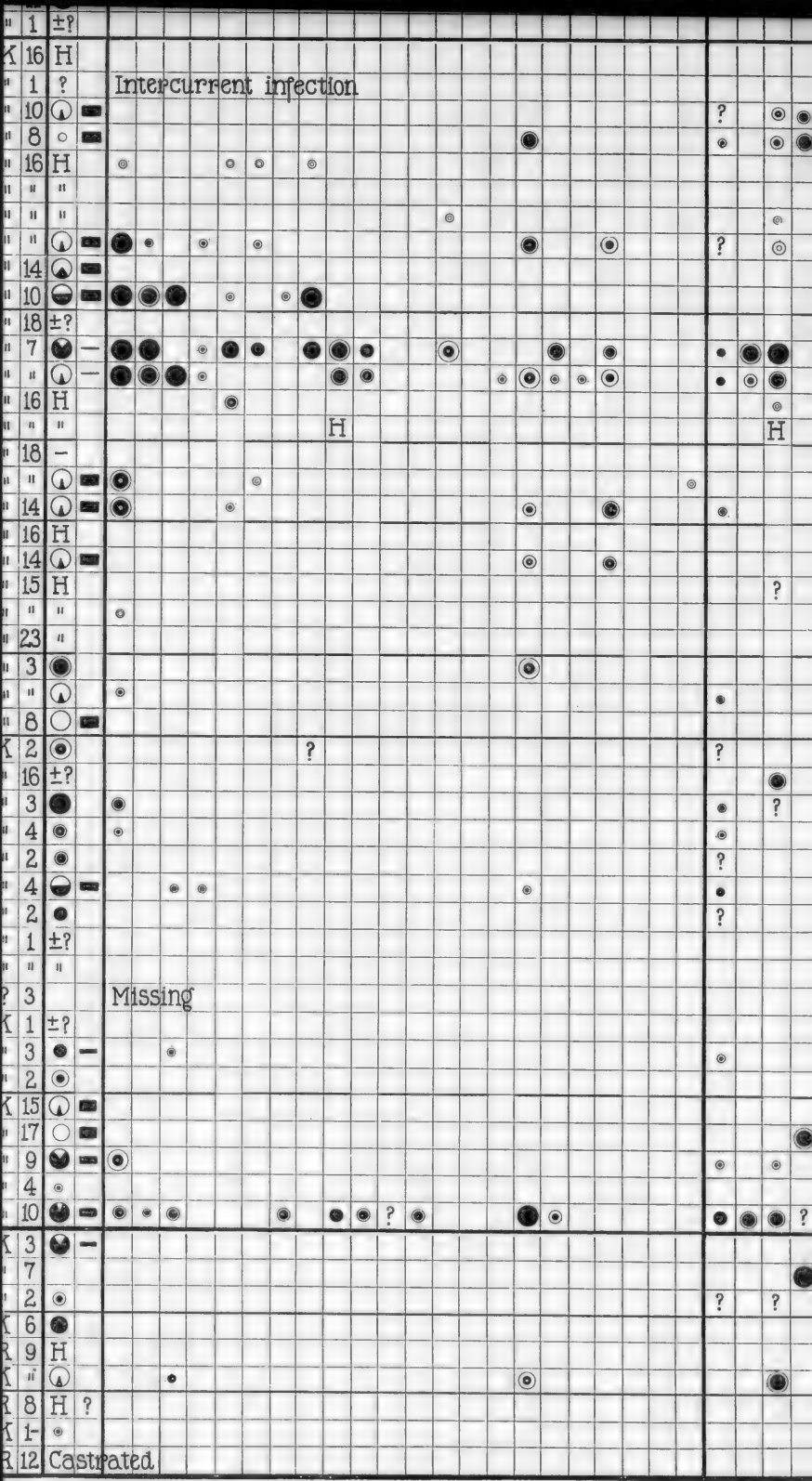
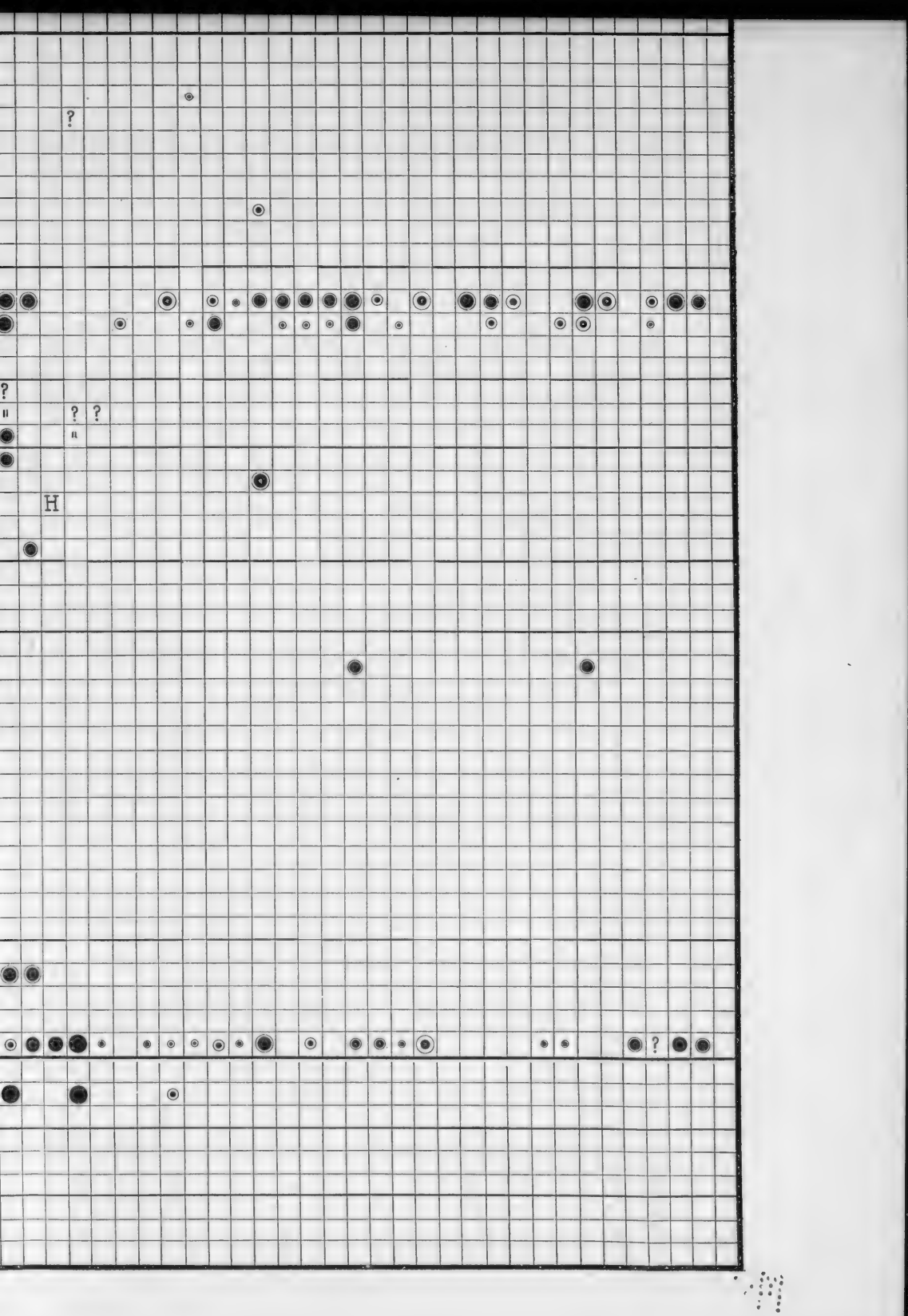


TEXT-FIG. 2. Tumor growth. Generations VII to IX.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CLINICAL

POSTMORTE

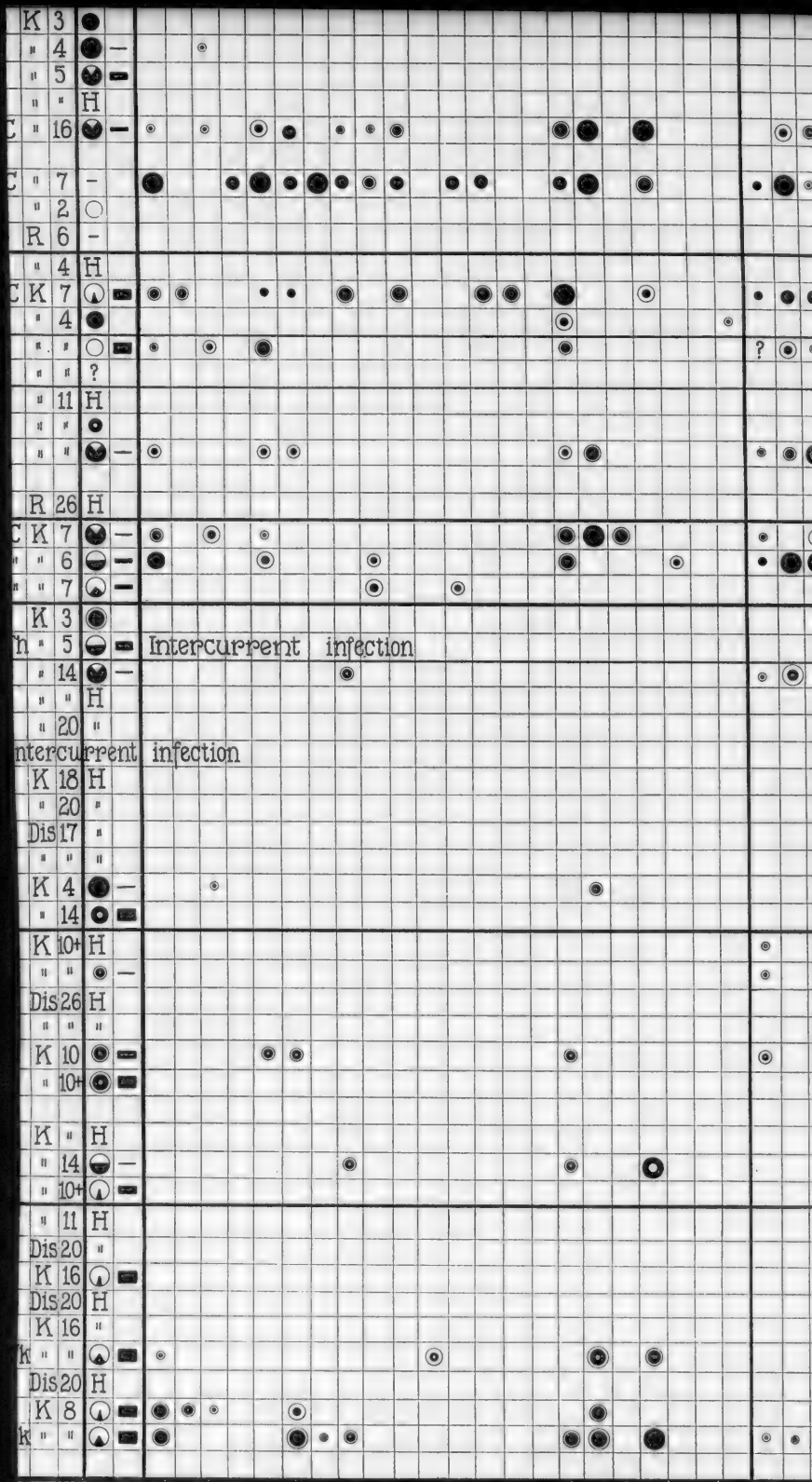
	Generation	Series	Date of inoculation	Animal number	Age group	Type	Primary tumor: Rate of growth	Maximum size	Time - weeks	Cord extension	Edema	Castrated	Recurrence	Metastases (Days after inoculation): R eyes	L eyes	Skin	Muscles	Bones	Subcutaneous tissues	Superficial lymph nodes: Inguinal-flank	Axillary-mammary	Cervical-submental	Uninoculated testicle	Condition	Termination	Time - weeks	Primary or recurrent tumor: State of growth	Encapsulation	R cords	L cords	Tunics	Scrotum
1921	X	A	12-6	1	II	F	<	◉	2																R	15	H					
				2	III	A	<	●	6																K	11	◔	▬				
		B	12-22	1	II	G	<<	●	2																"	9	○	▬				
				2	"	"	<<	●	"				Missing																			
1922		C	3-6	1	"	BlWBt	<<	●	"																"	2	●				◉	
				2	III	Br	-	-	-																R	7	-					
				3	IV	A	<	●	4																K	26	◔	▬				
				4	II	BlWBt	<<	◉	1																"	1	◉					
				5	III	A	<<	◉	"																"	"	◉					
				6	"	F	<<<	●	3																R	7	H					
				7	"	Hm	<<<	●	"																K	3	●					
				8	"	Br	<<<	●	4																"	4	●	—			◉	
				9	II	GT	<<<	●	"																"	5	◒	▬				
				10	III	NZ	<	◉	2?																"	"	H					
				11	"	F	<<<	●	3		21			121				114							C	"	16	◒	—	◉		◉	
				12	"	BrWBt	?	?	?	Intercurrent infection																							
				13	"	Bl	<<<	●	3			21													C	"	7	-		●			●
				14	II	G	<<<	◉	2		12															"	2	○					
				15	III	F	<<<	●	3			21														R	6	-					
	XI	A	3-27	1	III	Br	<	◉	?																"	4	H					
				2	"	Bt	<<<	●	7	◉															C	K	7	◔	▬	◉	◉		
				3	II	G	<<<	◉	4																"	4	●						
		B	4-10	1	"	Bd	<<<	●	3		21														"	"	○	▬	●		◉	
				2	III	Bl	?	±?																	"	"	?					
		C	7-5	1	"	Br																			"	11	H					
				2	II	BrW	<<	●																	"	"	◉					
				3	"	G	<<	●									70								"	"	◒	—	◉			
				4									Died during summer																			
				5	"	A																			R	26	H					
	XII		4-26	1	II	BlW	<<	●	7	◉				47				47						C	K	7	◒	—	◉		◉	
				2	III	Br	<<	●	4	●	19			42		42		42						"	"	6	◒	—	●			
				3	"	"	<<	●	7																"	"	7	◔	—				
	XIII	A	6-7	1	II	NZ	<<	◉	2+																	K	3	◉					
				2			<	◉	4																Th	"	5	◒	▬	Intercur			
		B	6-16	1	"	G	<	●	?																"	14	◒	—				
				2	III	"	<<	●	"																"	"	H					
				3	"	F	<<<	●	"																"	20	"					
				4	II	A	<	?	"				Died during summer												Intercurrent infection							
				5	III	Br	<	"	"																K	18	H					
				6	II		<	"	"																"	20	"					

POSTMORTEM

Condition	Termination	Time-Weeks	Primary or recurrent tumor: State of growth	Primary or recurrent tumor: Encapsulation	Extensions and implantations: R cords	L cords	Tunics	Scrotum	Omentum	Mesentery	Diaphragm	Parietal peritoneum	Abdominal fat	Intestines	Stomach	Pancreas	Bladder	Seminal vesicles	Abdominal walls	Retroperitoneal tissues	Deep lymph nodes: " & mesenteric	Deep lymph nodes: Peritracheal-peribronchial	Mediastinum	Parietal pleura	Pericardium	Epicardium	Lungs	Liver	Kidneys
	R	15	H																										
	K	11	◔	▬																									
	"	9	○	▬																									
	"	2	●				⊙					⊙																	
	R	7	-																										
	K	26	◔	▬																									
	"	1	⊙																										
	"	"	⊙																										
	R	7	H																										
	K	3	●																										
	"	4	●	—			⊙																						
	"	5	◒	▬																									
	"	"	H																										
C	"	16	◒	—	⊙		⊙		⊙	●		●	●	⊙						⊙	●		●					⊙	●
C	"	7	-		●				●	●	●	●	⊙	●		●	●			⊙	●		⊙				●	●	⊙
	"	2	○																										
	R	6	-																										
	"	4	H																										
C	K	7	◔	▬	⊙	⊙			•	•		⊙		⊙			⊙	⊙											
	"	4	●																	⊙			⊙				●	●	●
	"	"	○	▬	⊙		⊙		⊙											⊙						⊙			
	"	"	?																	⊙							?	⊙	●
	"	11	H																										
	"	"	⊙																										
	"	"	◒	—	⊙				⊙	⊙										⊙	⊙						●	⊙	●
	R	26	H																										
C	K	7	◒	—	⊙		⊙		⊙											⊙	⊙	⊙					●		⊙
"	"	6	◒	▬	●				⊙			⊙								⊙							•	●	●
"	"	7	◔	▬								⊙				⊙				⊙				⊙					
	K	3	◉																										
Th	"	5	◒	▬	Intercurrent infection																								
	"	14	◒	—								⊙															⊙	⊙	
	"	"	H																										
	"	20	"																										
Intercurrent infection																													
	K	18	H																										
	"	20	"																										

Distant metastases
R } suprarenals
L }
R } eyes
L }
Hypophysis
Pineal
Thyroid
Parathyroids
Cranial cavity
Spinal canal
Uninoculated testicle
Spleen
Thymus
Inguinal-flank
Axillary-mammary
Cervical-submaxillary
Facial
Calvarial
Spinal
Thoracic
Anterior extremities
Posterior "
" "
Anterior "
Shoulder & thoracic
Cervical
Facial
Abdominal walls
Lumbar
Heart
Skin
Subcutaneous tissues
Superficial lymph nodes
Bones
Muscles

| | | |



TEXT-FIG. 3. Tumor growth. Generations X to XV.

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CLINICAL

POSTMORT

Generation	Series	Date of inoculation	Animal number	Age group	Type		Primary tumor: Rate of growth	Maximum size	Time - weeks	Cord extension	Edema	Castrated } days	Recurrence	Metastases (Days after inoculation): R } eyes	L }	Skin	Muscles	Bones	Subcutaneous tissues	Superficial lymph nodes: Inguinal-flank	Axillary-mammary	Cervical-submental	Uninoculated testicle	Condition	Termination	Time - weeks	Primary or recurrent tumor: State of growth	Encapsulation	R } cords	L }	Tunics	Gen
XVI	A	9-12	1	II	Br		<	◉	2+																K	3	◕	—				
			2	"	G		<	●	2-																"	2-	●	—				
			3	III	F		<	◉	3-		20														"	3	◯	▬				
	B	10-13	1	II	F	R	<	◉	4																K	9+	◎	▬				
		10-23				L	<	◉	"																"	"	◎	▬				
			2	"	B1		-	-	-																"	"	◑	▬				
							<	◉	2																							
			3	"	GF		<	◉	6																"	8	◑	▬	●	●		
							<	◉	4																							
			4	"	F		-	-	-																"	9	-	▬				
							<	◉	3																		H					
			5	"	B1		-	-	-																"	8	-					
							<	◉	2																		H					
			6	"	GF	R	Intercurrent infection scrotum																		"	2+						
			7	"	F		<	◉	6																"	9+	◑	▬				
							<	◉	4																		◯					
			8	"	Br		±?	?	?																"	"	±?					
							"	"	"																		"					
			9	"	"		<	◉	6																"	8	◯	▬				
							<	◉	4																		○					
			10			R	?	±?	1-																D	1-	±?					
			11	"	BrG		<	●	3																K	9+	◑	▬				
							<	◉	4		17	28															Castrated					
			12	"	F		-	-	-																"	"	-					
							<	◉	4																		◎	▬				
XVII		9-25	1	II	Br		<	◉	1		7														K	8	H					
			2	"	BrG		<	◉	4	?															"	4	◕	—				
			3	"	G		<	◉	"		7														"	8	◉	▬				
			4	"	Br		-	-	-																"	10	-					
			5	"	GBr		<	◉	3																"	3			●			
			6	"	BrW		<	◉	2																"	8	◎	▬				
			7	III	G		-	-	-																"	10	-					
			8	"	BrW		-	-	-																"	3	-					
			9	"	G		<	◉	3																"	8	◯	▬				
			10	"	Br		-	-	-																"	2+	-					
			11	"	M		<	Retr. abd. cavity							55										C	"	8	◕	▬				
			12	"	NZ		<	◉	3																Dis	3	◉					
XVIII		10-23	1	II	G		<	◉	4																K	12	○	▬				
			2	"	B1		<	◉	5																"	"	◑	▬	•			
			3	"	G		<	◉	2																"	"	H					
			4	"	Br		<	◉	4	◉															"	"	H					
			5	"	F		<	◉	7	◉															C	D	8	◑	▬			

POSTMORTEM

Condition	Termination	Time-weeks	Primary or recurrent tumor: State of growth	Primary or recurrent tumor: Encapsulation	Extensions and implantations: R cords	L cords	Tunics	Scrotum	Omentum	Mesentery	Diaphragm	Parietal peritoneum	Abdominal fat	Intestines	Stomach	Pancreas	Bladder	Seminal vesicles	Abdominal walls	Retroperitoneal tissues	" & mesenteric	Deep lymph nodes: Peritracheal-peribronchial	Mediastinum	Parietal pleura	Pericardium	Epicardium
K	3		◕																							
"	2-		●																							
"	3		○																							
K	9+		◎																	⊙	⊙		⊙			
"	"		◎																							
"	"		◔										●		?						⊙					
"	8		◕		⊙	⊙																				
"	9		◕																							
			H																							
"	8		-																							
			H																							
"	2+																									
"	9+		◔											⊙	⊙						⊙					
			◯																							
"	"		±?																							
			"																							
"	8		○																							
			○																							
D	1-		±?																							
K	9+		◔																							
			Castrated																							
"	"		-																							
			◎																							
K	8		H																	◎						
"	4		◕																		⊙					
"	8		●										●													
"	10		-																							
"	3				⊙																⊙					
"	8		◎																							
"	10		-																							
"	3		-																							
"	8		○																							
"	2+		-																							
C "	8		◕						⊙	⊙	⊙									⊙	⊙		⊙			
Dis	3		⊙																							
K	12		○																							
"	"		◔		⊙																⊙					
"	"		H																							
"	"		H																							
C D	8		◯																	⊙						

Uninoculated testicle

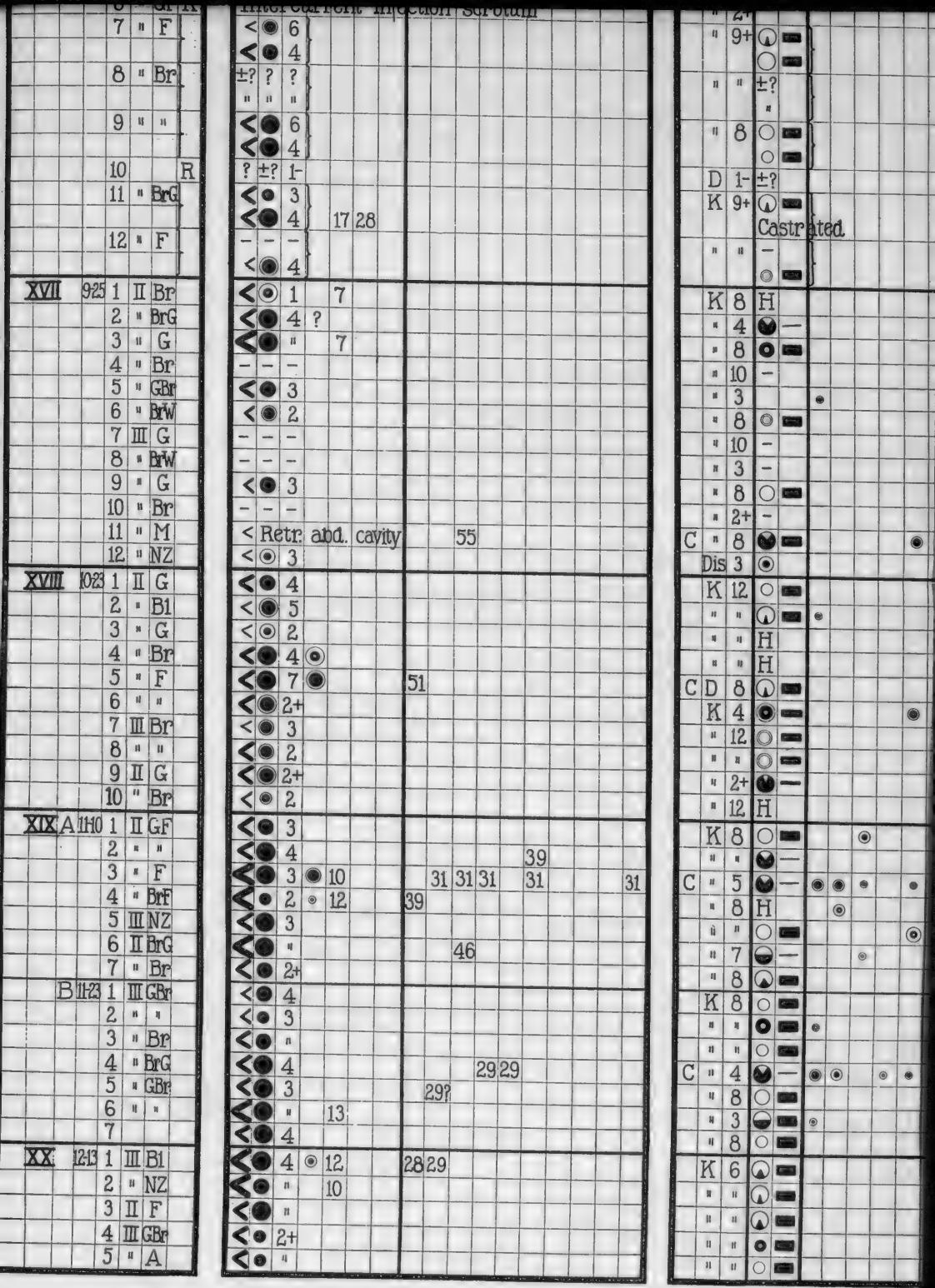
Distant metastases

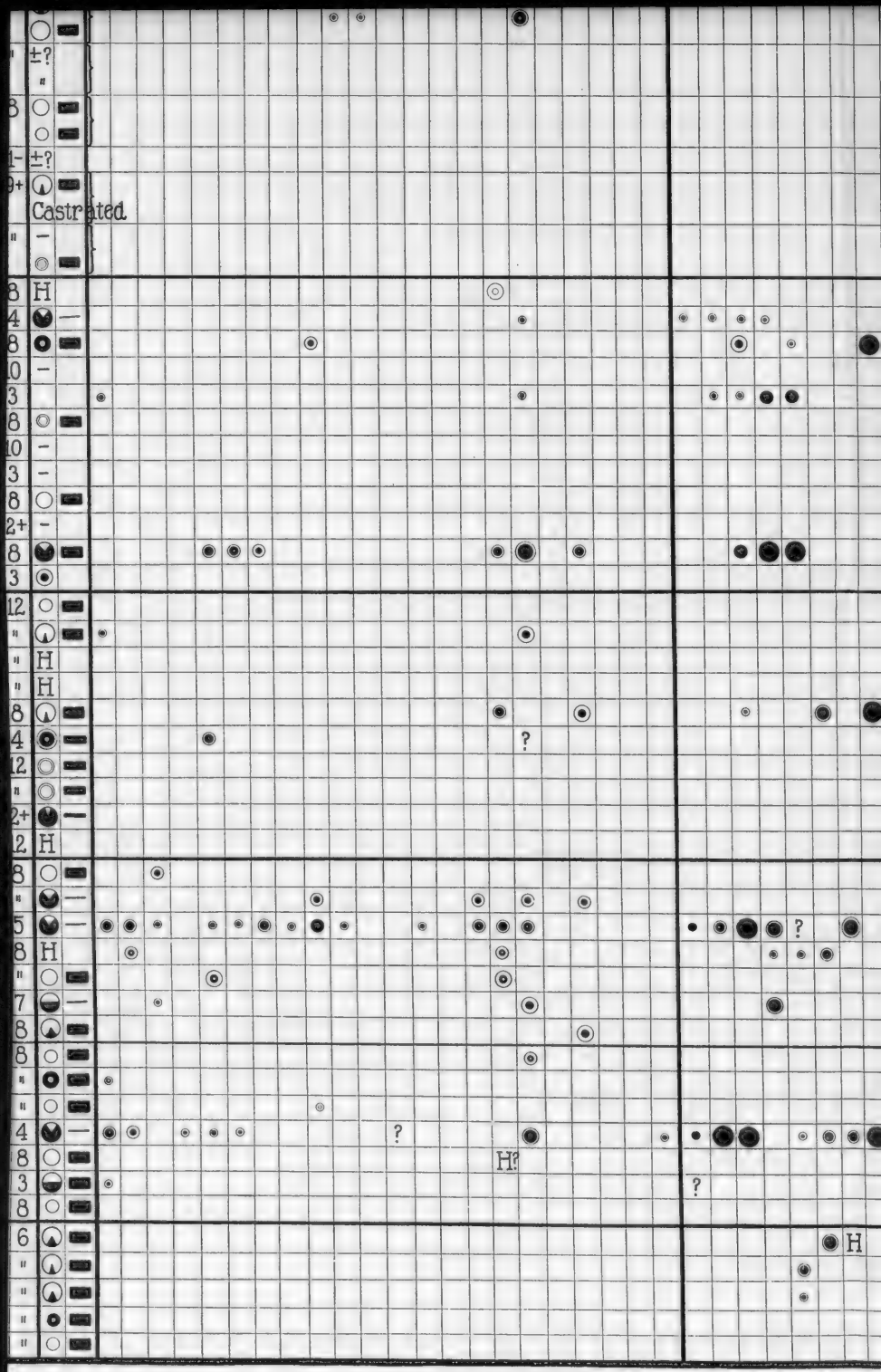
Superficial lymph nodes

Bones

Muscles

Lungs
Liver
Kidneys
R } suprarenals
L }
R } eyes
L }
Hypophysis
Pineal
Thyroid
Parathyroids
Cranial cavity
Spinal canal
Uninoculated testicle
Spleen
Thymus
Inguinal-flank
Axillary-mammary
Cervical-submaxillary
Facial
Calvarial
Spinal
Thoracic
Anterior extremities
Posterior "
" "
Anterior "
Shoulder & thoracic
Cervical
Facial
Abdominal walls
Lumbar
Heart
Skin
Subcutaneous tissues


Intercalation Infection Scrotum
Castrated.
XVII
XVIII
XIX
XX
Retr. abd. cavity
55
Text-Fi



TEXT-FIG. 4. Tumor growth. Generations XVI to XX.

data as to the number, kind, and distribution of secondary tumor growths divided on the basis of extensions or implantations and metastases to distant parts of the body.

The symbols and abbreviations used in these charts will require some explanation. In the first place, the animals were divided into four age groups on the basis of development since, in most instances, the age of the animal was unknown. These groups may be designated as young, young adult, adult, and old, which are indicated by the use of the Roman numerals I, II, III, and IV respectively. Group I includes animals that had not reached sexual maturity at the time of inoculation; Group II, animals that were sexually mature but had not attained full development; Group III, animals that were fully developed and in the prime of life; while Group IV includes animals in which senile changes were apparent.

A second classification of animals was made on the basis of breed or color markings. As far as possible, the breed or predominant cross is indicated on the chart by a system of abbreviations (see Explanation of Text-figs. 1 to 4). In other instances, the type of the animal is indicated by color markings or by a combination of the two designations. Thus, the abbreviation Bg. indicates a Belgian hare or an obvious cross of this breed as distinguished from common brown or gray-brown rabbits which are designated on the chart by the abbreviations Br. or G.Br. respectively. In the latter instance, it will be noted that the abbreviation used is a combination of the abbreviations for gray and for brown and will serve as an example of the system of combination that is used throughout.

The rate of growth of primary tumors is graded by the use of appropriate symbols as slow, moderate, active, or very active. As used here, these terms refer to conditions that prevailed during the first 3 or 4 weeks after the tumor became palpable. Rate of growth is not to be confused with the maximum size attained by the tumor nor even with the time within which the maximum size was attained, although in most instances, there is a close agreement between these two conditions. As a rule, estimations of the rate of growth were based on clinical observations rather than on actual measurements, and allowance must be made for an unavoidable change in standards of evaluation as the rate of growth increased with successive generations. This applies chiefly to an attempt to compare rate of growth at the beginning and towards the end of this series of experiments. The difference was greater than the symbols would indicate.

The size and state of both primary and secondary tumors as well as the degree of involvement of particular organs or tissues are represented by a system of circles. These symbols are divisible into three major groups, depending upon whether the tumor was in an active condition and composed largely or entirely of living tissue; whether it was inactive or regressing; or was largely necrotic and in process of resolution and absorption, or healing. Tumors that had completely healed are designated by the letter H.

Active tumors are represented by circles with a solid black center (Group I). These are the basic symbols of the system and they are subdivided into four sizes which represent the relative size of individual tumor nodules, the size increasing from 1 to 4. A further subdivision is made on the basis of the relative number of nodules or the extent of involvement of a particular organ or tissue which is indicated by the size or portion of the circle that is black, the number increasing from A to C. Thus, the symbols marked 4A, 4B, and 4C all indicate the presence of large nodules or of nodules that were large in proportion to the size of the organ, but in the first instance there would be only a few nodules or only a small portion of an organ would be occupied by tumor growth while in the last instance there would be a large number of isolated nodules or a large part of the organ would be taken up by the tumor. In like manner, the symbols 1, 2, 3, and 4 of any one group A, B, or C indicate an equal number of nodules or an equal degree of involvement, the only difference being the size of the nodules present. Thus, the extent of the growth indicated by 1C and 4C is the same, but in one case the growth would be composed of numerous small nodules while in the other there might be a single large mass filling the greater part of an organ, or a number of large nodules.

Two exceptions to this general rule are to be noted. In the case of primary tumors, the idea of numbers could be dispensed with, while it was necessary to make a greater allowance for possible size variations with relation to the size of the testicle so that the entire series of symbols included in Group I was employed to express this relationship. Here the increase in size runs from A to C and from 1 to 4 in each of these classes so that the smallest recognizable growth would be represented by the symbol marked A1 and the largest by C4 while any B tumor would be larger than any A tumor and C larger than B.

Another difficulty was encountered in the classification of metastases in such organs or tissues as the skin, subcutaneous tissues, muscles, and bones which were never involved to an extent equal to that of other organs. In order to meet this situation the highest values were assigned to the largest or most extensive growths that were encountered, while other lesions were graded with relation to these.

Tumors that were inactive or regressing are represented by the introduction of a white center into the same system of circles, while those that are in process of healing have the black center replaced by a second circle within the first or are represented by a single circle in the case of tumors corresponding to those of the C class in Group I.

An addition to this system is made in the case of autopsy records of primary or recurrent tumors. Here the proportions of living and necrotic tissue in large tumors (C4 tumors) that still retained some growth activity are shown by the portions of the circle that are represented in black and white respectively.

The degree of encapsulation of primary tumors is indicated by a series of black bars of varying widths which correspond with the relative thickness and density of the capsule.

The abbreviations used with reference to the terminal condition of animals have a special significance. In the first place, no attempt was made to record the condition of an animal unless it was obviously and decidedly abnormal. If the animal was extremely thin or emaciated but apparently strong and active it was recorded as thin (Th.); if, on the other hand, the animal was well nourished but extremely weak it was recorded Wk. When both conditions existed and were associated with marked pallor of the mucous membranes the animal was designated as cachectic (C.). The abbreviation Pr. (poor) was used to indicate a similar condition but less clearly defined as to weakness and pallor of mucous membranes; when there was complete prostration, and death appeared to be impending, the animal was classed as moribund (M.).

Other signs and abbreviations require very little explanation. The question mark (?) wherever used indicates a possible or doubtful lesion or condition. The plus-minus sign (±) has a similar significance but is used only to indicate uncertainty as to the results of an inoculation; when used separately these signs (+ or −) have their usual significance.

Comparison of clinical and postmortem data will reveal some apparent discrepancies, especially so in the case of secondary growths in the cord of the inoculated testicle. These differences are due to the fact that the two evaluations were made independent of each other and that the size or extent of involvement, as determined by clinical examination, appeared at times to be greater than that found on postmortem examination.

For convenient reference, we have inserted a brief record of the primary and secondary growth that occurred in the animal with the spontaneous tumor at the top of Text-fig. 1. In interpreting this record, it is to be noted that the primary tumor was excised 18 days after it was first seen, that there was a recurrence, and that the excision of this growth was followed by a second recurrence.

Results of Continued Transplantation.

In presenting the results of continued transplantation, no attempt will be made to consider each of the twenty generations in detail. The records that have been provided make it possible for anyone to undertake comparisons of this kind. It will serve the purposes of this report equally well if we limit ourselves to a general consideration of those features of the results that indicate most clearly what happened during this period of transplantation, but the discussion will be based largely on data recorded in Text-figs. 1 to 4 and for convenience will follow the general order observed in these charts.

Morphology of the Tumor.—Frequent examinations of both primary and secondary tumors have failed, thus far, to show any differences in the gross or microscopic appearance of the tumor that cannot

be accounted for on the basis of varying conditions of cellular activity. The general morphology of the cells and the structure of the growth appear to have been unaffected by transplantation.

Percentage of Takes.—With one exception, failure to obtain a primary growth by intratesticular inoculation has occurred so rarely as to be of very little significance. It will be noted that in the majority of instances in which a negative or an uncertain result is recorded, the animal lived a very short time after inoculation or else there was some complicating condition, such as a secondary infection, to obscure the result. A good illustration of this point is supplied by the second animal in Generation VIII B. In this instance, there was apparently no growth in the testicle and the animal was regarded as negative until a fungating tumor mass was detected in the lower jaw nearly 4 months after inoculation.

The important point to be noted with reference to the percentage of takes is that the tumor grew in three of the first four rabbits inoculated from metastatic nodules in the liver of the original animal, and that thereafter, with the exception of one brief period in the fall of 1922 (Generations XVI and XVII), there was only an occasional instance in which a definite growth was not obtained. In the seventeenth generation the tumor failed to grow in four out of twelve rabbits. A second series of twelve rabbits was then inoculated with material taken from an earlier generation, constituting Series B of Generation XVI, with less than 50 per cent positive results (five out of twelve). Still, a second inoculation (left testicle) 10 days later gave positive results in all but one of the ten animals that were reinoculated.

This experience might be attributed to the use of poor or damaged material or to some error in technique, were it not for the fact that there is no other evidence to show that such results might be produced in this way, and that the high incidence of negative results harmonizes better with what is known concerning the general behavior of the tumor at this particular time.

On the other hand, the high percentage of negative results obtained in the sixteenth and seventeenth generations cannot be regarded as an effect of transplantation, since there has been no repetition of this condition. In general, the percentage of takes, even at the present

time, is essentially the same as during the early generations. Still, there has been a definite increase in the proportion of successful inoculations by the use of less favorable methods than the intratesticular route.

Incubation Period.—During the first two or three generations it required from 9 to 14 days to produce a palpable tumor in the testicle, but with successive transfers there was a gradual reduction of this time until the incubation period rarely exceeded 5 to 7 days and the initiation of the growth became merged with the reaction to inoculation so that no exact time could be fixed. Still, the incubation period has varied with different series and is by no means constant.

Growth and Persistence of Primary Tumors.—The most significant features of the primary tumor are the rate and character of the growth, the size attained, the degree of encapsulation, and the ultimate fate of the tumor. These conditions can best be considered in relation to one another.

On the whole, it can be said that none of these conditions exhibited a steadily progressive tendency in any given direction. During the second generation and the first part of the third, primary tumors attained a high rate of growth. But the rate of growth varied first in one direction and then in another, until it reached a very low level in the thirteenth generation and remained comparatively low until the nineteenth generation, when there was a decided change in the direction of an increased rate of growth.

In like manner, there were decided variations in the mode of growth of primary tumors and the size attained by the average tumor within a given period of time or the proportion of tumors that reached a large size within the first few weeks after inoculation. At the beginning of transplantation the tumor tended to form irregular nodular masses which required upwards of 7 to 10 weeks to attain their maximum size (Generations I and II). Later, the tumor grew more diffusely and reached a large size within 4 to 6 weeks, and eventually within 3 to 4 weeks. The tendency to a more vigorous growth was apparent as early as the second and third generations but was not maintained at a constant level. It will be noted, however, that toward the end of this series of inoculations (Generations XIX and XX), all tumors were of comparatively large size and attained their maximum growth

within a period of 2 to 4 weeks, which is in striking contrast with the results obtained in the first two generations.

If we consider the state of the primary tumor at the time of autopsy, we will find additional evidence of the occurrence of changes in the growth activity of tumor cells. In this instance, we have to consider the survival of tumor cells and the resolution and healing of primary tumors. Among the animals of the earlier generations there was a greater tendency to the persistence of primary tumors with a larger proportion of living tissue than among those held for corresponding periods of time in later generations. This may be seen by comparing the results given for Generations II and VI with those for the last three generations. There was no instance of complete healing of a primary tumor in the first or second generation, although eleven of the thirteen rabbits were held for a minimum of 8 weeks, while five of them were kept under observation for upwards of 6 to 7 months. Two animals showed tumors in process of healing at the expiration of 24 weeks but one of these never developed a large tumor, while the other had been castrated and the growth present represented a recurrence (Generation II, Nos. 1 and 6).

From the third generation onward, complete healing of primary tumors occurred in a variable proportion of animals of nearly all the generations in which the average period of observation exceeded 4 weeks and not infrequently reached as high an incidence as 40 to 50 per cent among animals that were held as long as 12 to 16 weeks. The time of healing is not given in the charts and the period of observation was so irregular that an exact comparison of the results for different generations cannot be made. Nevertheless, it may be said that while the proportion of tumors that healed within a specified period of time varied greatly, there was a decided tendency for resolution and healing to occur earlier as transplantation was continued. Similar conditions obtained with reference to encapsulation.

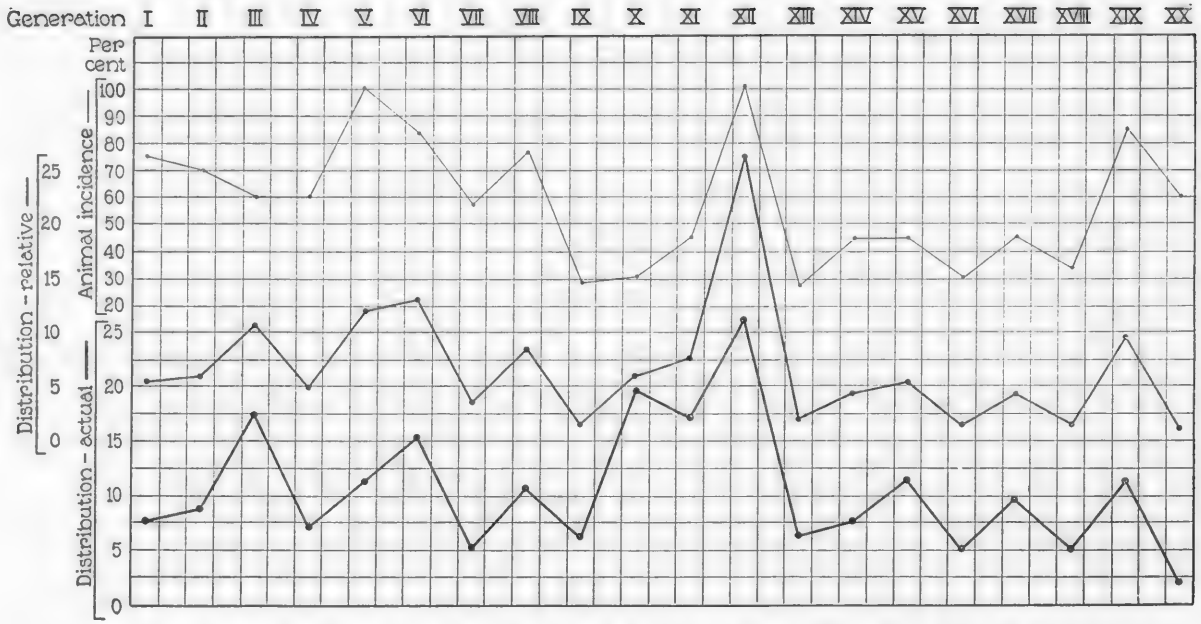
In so far as the growth and persistence of the primary tumor are concerned, it would appear, therefore, that continued transplantation produced two effects; an increased rate of growth and an increased extent of growth which were in turn counterbalanced by a shortening of the period of active growth and an increase in the rate and extent of the local reaction on the part of the animal, all of which had the effect

of hastening the course of events following inoculation whether they tended towards recovery or the extension of the growth to other parts of the body. This will be brought out more clearly by a consideration of the effects of transplantation on the occurrence and distribution of secondary tumors.

Incidence and Percentage Distribution of Secondary Tumors.—The phenomena of metastasis or of secondary tumor growth give a far better conception of the behavior of this neoplasm than can be gained from a study of the primary tumors. In order to simplify analysis of this phase of the subject the data contained in Text-figs. 1 to 4 have been reduced to a series of curves (Text-fig. 5) which show the proportion of animals of each generation in which secondary tumors were observed clinically or at autopsy and the distribution of these lesions. These curves will be referred to as the curves of incidence and distribution. The distribution curves, as here given, are based on the number of organs or tissues in which tumors were found as compared with a theoretical possible distribution which was determined by the actual location of secondary tumors during the first twenty generations. The first of these curves (relative distribution) gives values calculated on the basis of all animals of the group, while the second curve (actual distribution) includes only those in which secondary tumors were present.

In making the calculations for the curves of incidence and distribution we eliminated all animals that were held less than 2 weeks and all of those that were missing or lost. Healed tumors were taken into account but doubtful or uncertain lesions were regarded as negative.

It is to be noted that the curve of incidence indicates merely the liability to the occurrence of secondary growths among the animals of a given group, while the distribution curves give some conception of the actual prevalence of metastases and the extent of the growth in these animals and is, therefore, a more accurate indicator of the malignancy of the tumor. Still, the fact should be emphasized that there are conditions under which neither of these curves furnishes an accurate index of malignancy. This is especially true when conditions are such as to favor an initial development of metastases and the subsequent occurrence of resolution and healing. Under these circum-


Generation I II III IV V VI VII VIII IX X XI XII XIII XIV XV XVI XVII XVIII XIX XX
Per cent
Animal incidence
100 90 80 70 60 50 40 30 20
Distribution - relative
25 20 15 10 5 0
Distribution - actual
25 20 15 10 5 0

TEXT-FIG. 5. Incidence and distribution of metastases for first twenty generations.

stances, high values might be given by both curves when in reality none of the animals was likely to die. This is clearly illustrated by Generation XIV.

By reference to Text-figs. 1 to 4, it will be seen that secondary tumors occurred in a large proportion of animals even at the beginning of transplantation. In fact, of the four rabbits comprising the first generation, a growth was obtained in three, and all of them developed secondary tumors. It will be noted, however, that none of these animals developed very extensive metastases even though they were held for periods of 14 and 26 weeks. From this point onward, the course of events was very irregular and can be followed best by combined use of Text-figs. 1 to 4 and Text-fig. 5. For a time, it appeared that metastases were becoming more frequent and that the disease was assuming more malignant proportions. There was a slight reduction in the number of animals that showed metastases but this was compensated for by an increased distribution.

The high point in this direction was reached with the fifth and sixth generations, but, in the seventh, the incidence and distribution of metastases dropped unexpectedly to a much lower level. With the next generation there was some recovery, but again the incidence and distribution of metastases diminished, reaching a point lower than any previously recorded and did not return to a condition approximating that of the fifth and sixth generations until the twelfth generation was reached.

The twelfth generation was represented by only three rabbits but all of them succumbed to a very malignant type of disease and the pictures presented by them were so nearly alike that there could be little or no doubt as to the true malignancy of the tumor at this time. Still, this accession of malignancy was only temporary. From the thirteenth to the eighteenth generation inclusive, the incidence figures were lower than at any time except the ninth, tenth, and eleventh generations. The distribution figures were also much lower than they had been but were less affected on the whole than those for animal incidence.

The nineteenth generation brought a decided increase in the number of animals showing metastases and there was an appreciable increase in the distribution of the lesions, but the last generation of the

series showed a reduction in malignancy to a point lower than any that had been reached since the beginning of transplantation. Of the five animals comprising the twentieth generation, three developed metastases, but the growth was confined to the eyes of one animal and to the left suprarenal of the others. As was pointed out in a previous paper (5), the suprarenals, the hypophysis, and the eyes are the most poorly defended organs of the body, and metastases may develop in these organs with no probability of being able to extend to other parts of the body.

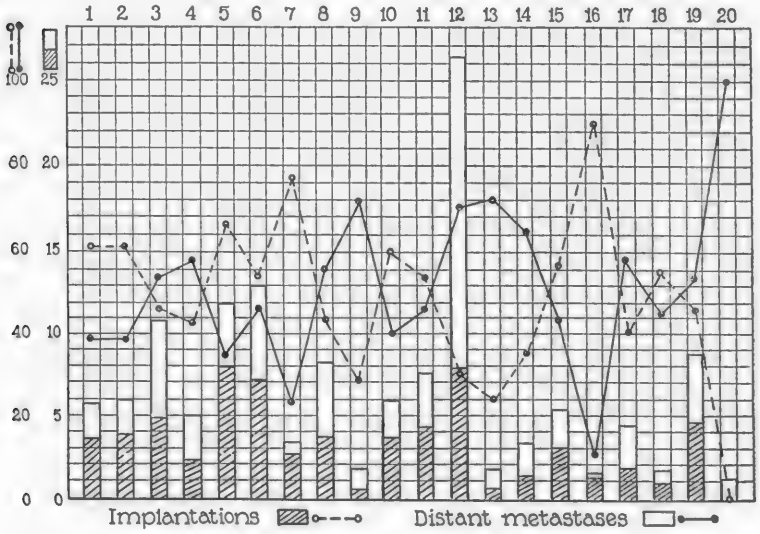
This brings us to a consideration of the character and location of secondary growths as influenced by transplantation.

Character and Location of Secondary Tumors.—By reference to Text-figs. 1 to 4, it will be seen that the character and location of secondary tumors changed fully as much during the first twenty generations as the incidence and percentage distribution of the lesions. At the beginning of transplantation, and for several generations thereafter, a large proportion of the secondary tumors belonged to the class designated as extensions and implantations. The quantitative relationships that obtained between lesions of this class and metastases to distant organs are shown in Text-fig. 6.

This figure shows the relative distribution of secondary tumors of all classes, which is indicated by the total height of the columns, and, in addition, the proportion of the lesions classed as extensions and implantations and metastases to distant organs without regard to the possible distribution in either case. It will be seen that at the beginning of transplantation, extensions and implantations were more frequent than metastases to distant organs. These relationships changed a number of times, but, during the first seven generations, the tendency was to increase the original disproportion between these two classes of lesions so that the most prominent feature of the disease during this period was the occurrence of implantations and the extension of the growth upward along the lines of the lymphatics from the region of the primary tumor. Gradually, this condition changed so that in later generations a greater proportion of the lesions occurred in organs or tissues far removed from the primary growth.

This change in the character and location of secondary tumors is clearly shown by a comparison of the conditions that prevailed dur-

ing the two periods of maximum malignancy in the fifth and sixth generations and in the twelfth generation. In the first instance, the great increase in secondary tumors was due largely to extensions and implantations, while in the twelfth generation it was attributable to a more widespread distribution of lesions. Only once was there a return to the condition that existed in the seventh generation. This was in the sixteenth generation when the malignancy of the tumor had reached an extremely low level.



TEXT-FIG. 6. Relative distribution of secondary tumors (twenty generations), showing the varying relations between implantation's and extensions and metastases to distant organs.

A similar change in the behavior of the tumor might be brought out by an analysis of the frequency and extent of involvement of different organs, but this aspect of the subject will be reserved for future consideration. It is sufficient to say that such a change has taken place, as can be seen by noting the gradual shift of metastases, as recorded in Text-figs. 1 to 4, from the first three columns including lungs, liver, and kidneys to a more general and more frequent involvement of organs placed further to the right.

Resolution and Healing of Secondary Tumors.—It will be noted (Text-figs. 1 to 4) that comparatively few secondary tumors are recorded as in process of resolution and healing and that fewer still are recorded as healed. Lesions of these classes have, however, a special significance and for that reason they will be considered briefly.

In the first generation three lesions are recorded as largely necrotic and apparently undergoing resolution at the expiration of 26 weeks. From the second to the fourth generation inclusive only one lesion was seen that appeared to be regresssing, although most of these animals were held for long periods of time.

Resolution and healing were first noted as conspicuous features of the disease among the animals of the fifth generation inoculated late in August, 1921. All of the animals in this group developed secondary growths, but, of the six animals in the series, three showed lesions that were largely necrotic and in process of resolution at the end of 8 weeks, while another showed a number of healed metastases in the kidneys 18 weeks after inoculation. The indications were that four of the six animals would have recovered.

In the next generation, there were fewer animals with retrogressing lesions, but, again, in the seventh and eighth generations the condition became more frequent, being associated here with some prolongation in the period of observation. From the ninth to the thirteenth generation, few instances of retrogression were seen, although there were a number of animals with secondary tumors that were held for a period of time comparable to that of the fifth and seventh generations.

In the fourteenth generation (July, 1922) necrosis and resolution were again a conspicuous condition in that at the end of 10 to 14 weeks all of the animals (four) that developed secondary tumors showed this condition as an outstanding feature of every lesion present. This tendency was carried over into the next two generations, occurring in two of the four animals of the fifteenth and three of the four in the sixteenth generation in which secondary tumors occurred. It then diminished in frequency until the nineteenth generation.

The tendency or liability to undergo resolution and healing has an important bearing on the estimations of malignancy based on the incidence and distribution of secondary tumors as stated above, but it has an additional significance in the present connection as evidence of a tendency to periodic changes in the behavior of the tumor.

Duration and Termination of Disease.—The final points to be considered in this series of experiments concern the duration and the mode of termination of the disease, neither of which can be stated with absolute accuracy, as it was impracticable to extend the period of observation indefinitely. From the data available, it can be said, however, that, at the beginning of transplantation, the expectancy of life appeared to be relatively good. In the first generation, one animal survived 26 weeks and another 14 weeks, with no definite indication as to what the outcome would be, but with a strong probability that they might recover. The third animal of this group lived 26 weeks, but would have died as a result of a growth involving the bones of the lower jaw and interfering with mastication.

In the second generation, there was no death attributable to the tumor, but one animal showed pronounced weakness at the end of 10 weeks and was killed. Again, there were three rabbits that survived for periods of 24 and 29 weeks. One of these virtually recovered within this period of time, a second was in good physical condition but showed several actively growing tumors, while the third (No. 9) became weak and emaciated and probably would not have lived more than a few days.

In the third generation, there were two animals that died from intercurrent infections, four recovered completely, and there were four deaths from the tumor. These deaths occurred 8, 11, 19, and 23 weeks after inoculation. The other animal of this group (B 5) was killed at the end of 8 weeks, but probably would have died within a short time, as there were extensive metastases in various parts of the body.

This establishes a fair basis for consideration of the duration of the disease and the probable proportion of deaths or recoveries, both of which varied greatly with succeeding generations. As early as the fourth generation there were two deaths within 6 to 7 weeks after inoculation. In later generations, some animals with malignant tumors lived as long as 14 to 16 weeks before their condition became critical, but the probable length of life of such animals rarely exceeded 7 to 10 weeks. Eventually this period was reduced still further so that in the nineteenth generation there was one death at 5 weeks and

another at 4; more recently, deaths have been recorded as early as 3 weeks after inoculation and have been not uncommon at 4 and 5 weeks.

This increased rate of progress of the disease was not limited to animals with highly malignant tumors, but, as transplantation continued, it became a characteristic feature of the disease in all classes of animals. The critical period in the growth of the tumor was greatly diminished, so that, in the great majority of instances, death or complete recovery occurred or was determined within 4 to 6 weeks after inoculation as compared with a much longer and rather indefinite period of time during the first three generations.

The effect on the death rate was distinctly different. The probable mortality for the first three generations may be estimated roughly at from 40 to 60 per cent. It is certain that higher points were reached in the fifth, sixth, and twelfth generations. On the other hand, it is doubtful whether any of the animals of the seventh, fourteenth, sixteenth, or twentieth generation would have died. It is certain, therefore, that, with the exception of three generations, there was no definite increase in the probable mortality and it would appear that in most instances the tendency was in the opposite direction. Still, this is not to be interpreted as evidence of an actual reduction in growth capacity of the tumor cells, as the results of more recent generations show that the tumor may be fully as malignant as at any time in the past.

DISCUSSION.

The influence of various factors that may have contributed to the production of the results here reported will be considered in the second section of this paper, so that in concluding this report, a brief summary of the outstanding features of the results is all that is necessary.

In the first place, the experiments that have been carried out show that we are dealing with a tumor that may be transplanted indefinitely, and that, thus far, transplantation has produced no material change in the morphology of the tumor and no fundamental alteration in its biological properties. Still, it cannot be said that the biological properties of the tumor cells have remained unaltered. The incubation period, the rate of growth of primary tumors, the time required

to attain a maximum size, and the persistence of primary tumors were all affected in a manner that indicated a decided increase in the rate of cell growth and the progress of the disease towards death or recovery. The same was true of the occurrence of edema in association with primary tumors and of metastases that could be recognized clinically. In addition, the character and location of secondary tumors changed in a way that suggested an increased distribution of tumor cells and an increased capacity for growth in all parts of the body. Still, with the exception of a few generations, there was no corresponding increase in the occurrence or percentage distribution of metastases, and, while the progress of the disease was greatly hastened, the death rate remained almost unaffected or was actually diminished.

Finally, it is important to note that none of the changes recorded was of a steadily progressive character. At times it appeared that the malignancy of the tumor had been greatly increased by transplantation, but almost immediately there was a change to a relatively benign condition, so that at the conclusion of this series of experiments the outstanding features of the results were: first, an unmistakable increase in the growth activity of the tumor cells which was associated with a corresponding reduction in the duration of the disease; second, a diminished incidence and distribution of secondary tumors with a corresponding reduction in mortality.

This paradoxical situation is a very significant feature of the results of transplantation and points clearly to the operation of some factor other than continued passage as the decisive influence in determining the end-results. This aspect of the subject will be considered in the second section of this paper.

SUMMARY.

A report is given of the results obtained by intratesticular inoculation of a malignant tumor of the rabbit based on a study of the first twenty generations.

The subject is presented from the standpoint of variations in growth and malignancy as they occurred with continued transplantation. Essential features of the experimental conditions and of clinical and postmortem observations are condensed and recorded graphically.

On analyzing the results of these experiments, it was found that many changes had occurred in the behavior of the tumor during transplantation, but that the changes were of a very irregular character and as a rule did not proceed constantly in any given direction. Moreover, the evidence as to the effect of transplantation on the growth and malignancy of the tumor was contradictory in that there was a great deal of evidence to show that there had been a decided increase in the activity and capacity for growth on the part of the tumor cells. But, with the exception of a few generations, there was an apparent reduction in the incidence and percentage distribution of secondary tumors, while the death rate was unaltered or actually diminished.

It was believed that this paradoxical situation afforded a basis for an explanation of the results that had been obtained by transplantation.

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

VI. Variations in Growth and Malignancy of Transplanted Tumors.

Part 2. Factors Influencing the Results of Serial Transplantation.

By LOUISE PEARCE, M.D., WADE H. BROWN, M.D., and CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The results of serial transplantation of this tumor were presented in the first section of this paper (1) without reference to conditions that may have influenced the outcome. In taking up this aspect of the subject, we wish to state that, as transplantation progressed, there was unmistakable evidence of the development of a more vigorous growth and of a wider distribution of cells. But the most clearly defined effect of this change was to convert a slowly progressive condition into one that progressed much more rapidly. There was no corresponding gain in the incidence and distribution of metastases, nor was there an increase in the lethal effects of the tumor.

For several generations it did appear that the malignancy of the tumor was increasing, but, as transplantation continued, the results became extremely irregular, with the suggestion of a tendency to a rhythmical occurrence of periods of increasing and diminishing malignancy which occasionally reached very high or very low levels. Still, from the sixth to the twentieth generation there was a general decrease in malignancy in spite of the fact that the growth of the tumor was becoming more and more active, and the progress of the disease was more rapid.

This series of changes was conditioned upon serial transplantation and there is no doubt that adaptation to passage played an important

part in the production of the changes that occurred, but it is equally certain that the course of events was greatly influenced by other factors. Minor variations in the results might be accounted for on the basis of irregularities in technique, but some other explanation would have to be offered for the extreme variations in malignancy and the conflicting tendencies that developed. The technique employed and the general care of the animals varied very little, and there is nothing to indicate that factors of this kind had any material influence on the results. The three most likely sources of variation in these experiments concern the material used for inoculation, the animals used, and the time at which the experiments were carried out, and we will limit ourselves to a consideration of these three factors. It should be borne in mind that we are not dealing directly with the general problem of the effect that may be produced by intentional efforts to vary these three conditions but the effect that may be produced when an effort is made to preserve uniformity of conditions; that is, with unavoidable variations in experimental conditions.

Material Used for Inoculation.

The extent to which the results of serial transplantation may have been affected by inequalities in the growth capacity of the material used for the inoculation of the different groups of animals is extremely difficult to determine. Realizing that this factor might be of considerable importance, we gave the greatest care to the selection of material so that any inequalities that may have existed were due to circumstances that were beyond our control.

Comparisons of the results obtained with material derived from different sources may be made in nine of the twenty generations included in this series of experiments as shown in Text-figs. 1 to 4, Part 1 of this paper, but in most instances the groups of animals are too small to have more than a suggestive value or there were other differences in experimental conditions which introduce an element of uncertainty into an interpretation of the results. This applies especially to the factor of time. The best opportunities for studying the influence of the material used for inoculation are afforded by the third and nineteenth generations and by the seventh and eighth generations.

Both the third and the nineteenth generations contained two groups of animals (A and B); the groups were of equal size and were inoculated with freshly prepared cell emulsions, the second groups receiving the material 16 and 13 days respectively after the first. In the third generation, the primary tumors grew more actively in Group A than in Group B. Still, the incidence of metastases was the same with a slightly wider distribution in Group B so that, in the end, there was no material difference in the results obtained in the two groups of animals.

In the nineteenth generation, the tumor was decidedly more malignant in one group of animals than in the other. It will be noted, however, that there was a closer agreement between the two subdivisions of this generation than with either the preceding or following generations (Text-fig. 4 of Part 1). In fact, the malignancy of the tumor appears to have increased to a maximum level in the first half of the nineteenth generation and then to have diminished (Text-fig. 5 of Part 1). It is uncertain, therefore, whether the results obtained in this instance are referable to the material used for inoculation or to some other factor that may have influenced the growth of the tumor at this time, independent of the material used.

This possibility is strengthened somewhat by results obtained with material that had been subjected to the supposedly deleterious influence of freezing, thawing, and grinding in the frozen state before being used for inoculation. In the seventh generation, results obtained with material treated in this manner can be compared directly with those obtained with an emulsion of the fresh material. In the eighth generation, the comparison is less satisfactory, as only a few of the control animals (Group B) were held even as long as 3 or 4 weeks. We can, however, make an indirect comparison with the first ten animals of Group A, and this gives an additional advantage of indicating the character of the results that were obtained at nearly the same time with material derived from still another source.

Fortunately, we have in this instance one generation (the seventh) in which the tumor was relatively benign, to compare with another in which it was distinctly more malignant, but this appears to have made no appreciable difference in the results in so far as the influence of the material is concerned. In the seventh generation, the results were

practically identical in the two groups of animals, showing that the treatment to which the material had been subjected had no appreciable effect on the character of the disease produced. The differences that appeared in the eighth generation were again comparatively slight. Metastases developed in three of the five animals inoculated with material that had been subjected to freezing, thawing, and grinding as compared with metastases in all of those (five) of the control group that were held as long as 3 weeks, and in six of the nine animals of Group A. The character and extent of the growth that might have developed in the animals of Group B are uncertain, but, in Group C, the malignancy of the tumor was fully equal to that shown by the animals of Group A (see Text-fig. 1, Series of November 1 and 9).

The evidence supplied by an analysis of the results obtained in these few cases is by no means conclusive. It is sufficient, however, to make it appear very unlikely that chance inequalities of the material used in this particular series of experiments were a factor of any considerable importance, and it is reasonably certain that the extreme variations in malignancy could not be accounted for on this basis.

Animals.

In considering the animals used in different experiments as a factor that may have influenced the results of transplantation, we are not concerned with the general problem of animal resistance or with individual peculiarities but with recognizable class distinctions as determined by such conditions as age or breed, both of which have been regarded as factors of considerable importance in the transplantation of tumors of other animals. On account of the irregular distribution of animals of different ages and breeds, it will be necessary to approach this problem from the standpoint of a comparison of results in general rather than by a systematic analysis of results from generation to generation.

Age.—Nearly all of the animals used in this series of experiments were classed as adults or young adults, so that the extremes of age played no part in the determination of the results. It will be of advantage, however, if we begin our consideration of the age factor by a brief reference to certain peculiarities of the growth of the tumor

in young and in old animals as illustrated by the eighth generation (see Text-fig. 2 of Part 1).

Group A of the eighth generation contained five rabbits 2 to 3 months old, seven young adults, seven adults, and three old animals that gave positive results on inoculation and were observed for a period of 3 weeks or longer. The young animals of this group showed an unusually rapid growth of primary tumors, and all of them developed secondary lesions of some kind. Still, the growth of the primary tumors was of relatively short duration, and at the conclusion of the experiment healing was complete in four of the five animals and in the other the tumor was almost entirely necrotic. The systemic distribution of lesions was also peculiar in that the growth was confined to the spermatic cord of the inoculated testicle (one animal), the deep lymphatics (one animal), and the eyes and suprarenal glands. None of these rabbits developed extensive lesions; in fact, there was only one animal with more than a single focus of secondary tumor growth, and with two exceptions all of the tumors were either healed or in the process of resolution at the time of autopsy. There was one metastasis in a suprarenal gland and one in an eye that remained active for periods of 16 and 23 weeks respectively, but neither of these animals showed living tumor tissue elsewhere; they were in excellent physical condition and in spite of the persistence of the growth in these locations it is practically certain that they would have recovered.

The peculiar response observed in this instance appears to be characteristic of the reaction of young animals, as similar results have been obtained on several occasions. It seems, therefore, that while the tumor grows exceptionally well in young animals, the resistance of such animals is of an equally high order, and that in a large proportion of cases the tumor will be of a relatively benign character.

Our experience with old animals has been very limited. There were only five rabbits in this entire series of experiments that were classed as old, and only three of these showed well developed senile changes. These are listed as "old" in Generation VIII A. The two others are No. 6 of the same generation and No. 3 of Generation X C. The results shown by these animals agree, in general, with those obtained in more recent generations.

The impression gained from a study of a small group of old animals is that the growth of primary and secondary tumors is comparatively slow but tends to be persistent, with an absence of the vigorous reaction and clear definition of results that are seen in young animals. Primary tumors may reach a very large size and there is usually some extension of the growth to other parts of the body, chiefly along the lines of the regional lymphatics, but on, the whole, the disease rarely assumes highly malignant proportions. Still, both primary and secondary tumors remain active for long periods of time, leaving the end-result in doubt.

It would appear, therefore, that the situation presented by old animals is, in general, the reverse of that presented by young animals. The two agree in that neither is apt to develop a highly malignant type of disease, but in the one case this appears to be attributable to conditions that are unfavorable to the growth of the tumor rather than to an active opposition on the part of the host, while in the other an extremely active growth is more than counterbalanced by a prompt and vigorous reaction on the part of the animal.

The growth of the tumor in young adult and adult rabbits suggests a gradual transition with increasing age from the condition presented by the young animal to that of the old. Considering the four age groups, it may be said that both the energy of cell growth and the expression of resistance on the part of the animal diminished with increasing age, but with the beginning of this change resistance decreased for a time more rapidly than growth with the production of a reversal of the relationships obtaining at the two extremes of life. This change in relationships affected young adult and adult animals in such a way as to increase the malignancy of the tumor in both classes of animals, but the conditions presented differed somewhat in the two cases.

An analysis of the results obtained shows that, on the whole, the growth of the tumor was slightly more active in young adult than in adult animals, that the incidence of metastases was slightly higher (61 as compared with 50 per cent), and that there was a tendency to a more extensive distribution of lesions and an earlier termination of the disease. These differences are well illustrated by the results shown in Generation VIII A.

This general statement of the influence of the factor of age must be applied with caution. There is no doubt that the results of the experiments under consideration were influenced to some extent by this factor, but it is well to note that in some instances the adult animals of a group gave better results than the young adults, and that some of the lowest values were obtained in groups of animals composed chiefly of young adults.

Breed.—The factor of breed has also played some part in the transplantation of this tumor, but the extent to which it has influenced the results obtained in different series of experiments is uncertain.

The spontaneous tumor developed in an albino rabbit, but, contrary to what might have been expected, it was soon found that animals of this type were not as well adapted to the growth of the tumor as some others. This is clearly indicated by an analysis of the results obtained in the second and third generations. In this case, the gray, the brown, the black, and the Flemish rabbits showed a much higher incidence and a wider distribution of metastases than the albinos, but the results obtained with different types of animals varied somewhat from generation to generation and on several occasions albino rabbits gave results comparable with those of any other type of animal (Generations VI and VIII A). Nevertheless, if we consider the entire series of experiments, it will be found that the results were fairly consistent and that there were distinct differences in the susceptibility of different classes of animals. This can be shown in a very simple way by comparing the liability to metastasis which is not seriously affected by irregularities in the observation period.

If all the animals in which the results of inoculation were negative or doubtful and those that were observed less than 3 weeks are omitted, the liability to metastasis among animals of a type that was used in any considerable numbers was as follows:

	per cent
Black	80
Gray	73
Flemish	60
Brown	51
New Zealand	50
Albino	50

The average for this entire group of animals (87 positive and 72 negative) was 54.7 per cent, while an additional group of 21 rabbits of miscellaneous types gave an incidence of metastases amounting to 66.6 per cent, or a combined figure of 56 per cent for the entire group of 180 rabbits.

We are not disposed to make any broad generalizations on the basis of the results obtained in this small series of animals, but it is quite evident that the liability to metastasis tends to vary with the color of the animal and that, in general, resistance to the growth of the tumor tends to increase from black to white so that, all other conditions being equal, the factor of color or breed, — of which color is one expression,— might be of very great importance.

There is no doubt that this factor had an appreciable effect on the results of the experiments under consideration; it also appears that, under the conditions that prevailed, the effect was in the main to equalize any differences that might have been produced by other factors. It so happened that in no instance was there an especially favorable or unfavorable grouping of animals and that the great majority of the animals used were of types that gave results close to the average. Animals that might have made a greater difference were not only used in small numbers but they were widely scattered. In addition, it will be seen that the extreme limits of variation in the results obtained for different generations (70 per cent) far exceeded the maximum variation (30 per cent) that might be attributed to any color or breed, and that both the maximum and minimum figures for the incidence of metastases were well outside of the figures for various breeds of animals.

It would appear, therefore, that while the chance grouping of animals must have affected the results of serial transplantation to some extent, any effect that might have been produced in this way would not provide a satisfactory basis for an explanation of the periodic occurrence of extremely malignant tumors or tumors of a benign character, nor would it be possible, on this basis, to account for the decided decrease in malignancy that occurred during the later generations.

Time.

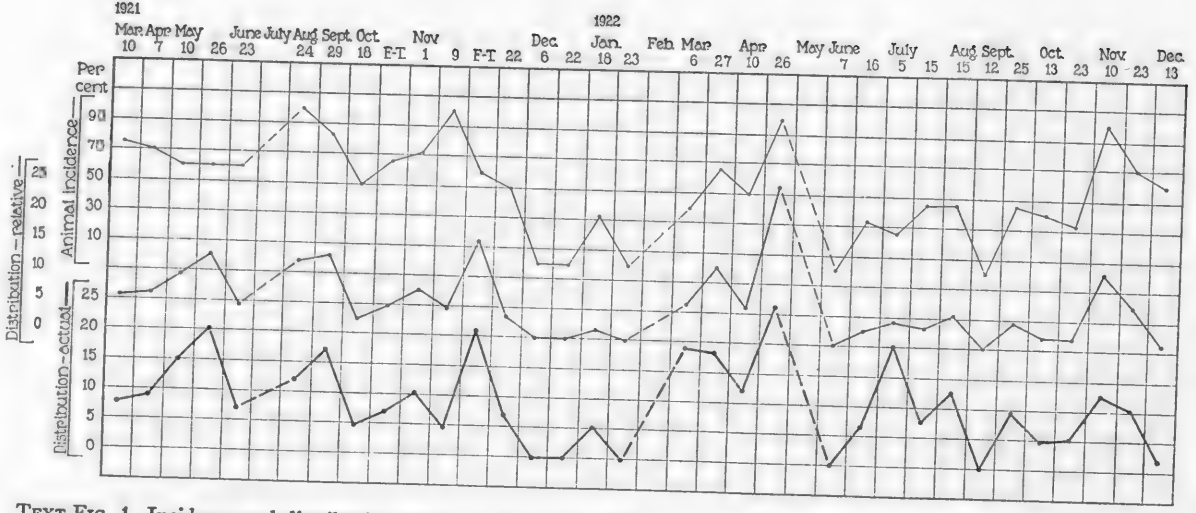
We come now to a consideration of the time at which the experiments were carried out as a factor that may have had some influence

on the behavior of the tumor. In order to facilitate an analysis of the results from this point of view, we have plotted two sets of curves (Text-figs. 1 and 2) showing the relative incidence of secondary tumors in successive groups of animals, and the percentage distribution of the lesions, calculated first on the total number of animals in the group (relative distribution) and then on the basis of those that were positive (actual distribution). Both sets of curves follow the time order of inoculation, irrespective of generations. In Text-fig. 1 the results are recorded for each series of animals, while in Text-fig. 2 we have combined these into monthly averages.

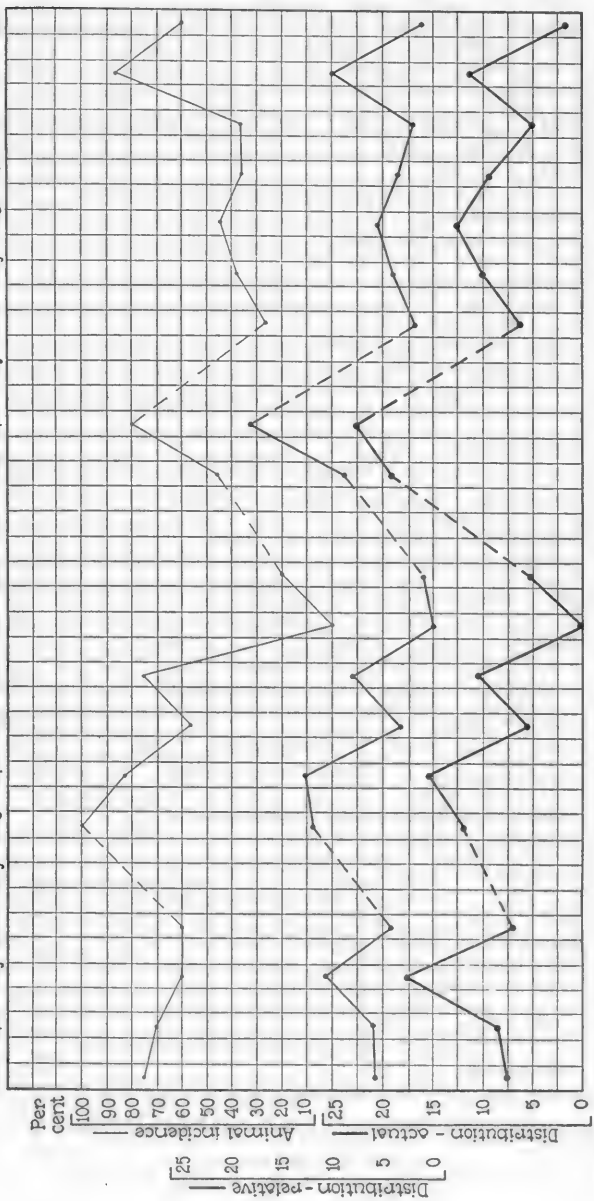
In analyzing these curves, it is well to bear in mind that the incidence curves indicate merely liability to metastases, while the curves of distribution give a more accurate conception of the severity of the disease. In general, the two sets of curves run parallel; but at transition points there is a tendency for a change in the direction of one curve to precede that in the others.

If we consider the incidence of metastases, as shown in Text-fig. 1, it will be seen that the first series of animals, inoculated on March 10, 1921, gave a slightly higher value than did any of those immediately following. Still, the curve remained relatively high through April, May, and June. There was a slight upward tendency in July (record not included in text-figures), but the first decided increase came in the group of animals inoculated late in August, and while the September series also showed a high incidence of metastases the curve shows a progressive reduction for September and October.

The October 18 series (Generation VII, Text-fig. 2 of Part 1) is especially interesting in that it was composed of two groups of animals, one of which was inoculated with material that had been subjected to freezing, thawing, and grinding before inoculation. The incubation period in this group of animals was about 2 weeks longer than that of the other group, so that from the standpoint of the growing time of the tumor this group stands about midway between the regular series of October 18 and November 1. It is interesting to note, therefore, that the results (distribution as well as incidence) show a similar relationship. From October to November 9 there was a progressive increase in the incidence of metastases followed by a marked reduction, which, we may say, persisted through the series of January 23,



TEXT-FIG. 1. Incidence and distribution of secondary tumors with relation to the time of inoculation. No data available for periods covered by broken lines. In this figure F.–T. indicates freezing and thawing.


1921
Mar Apr May June July Aug. Sept. Oct Nov Dec
1922
Jan Feb Mar Apr May June July Aug. Sept. Oct. Nov. Dec.
Per cent
100 90 80 70 60 50 40 30 20 10
Animal incidence
25 20 15 10 5 0
Distribution - actual
25 20 15 10 5 0
Distribution - relative

TEXT-FIG. 2. Incidence and distribution of secondary tumors considered on the basis of monthly averages in the order of inoculation. No data available for periods covered by broken lines.

1922, as the only slight elevation in the curve probably has no significance.

Here again attention may be called to the fact that different values were obtained for the two groups of animals inoculated on November 9. In this instance, the delayed development of primary tumors in the animals inoculated with material that had been frozen, thawed, etc., was associated with a lower incidence of metastases, which places this group of animals in a position between that of the regular series of November 9 and that of November 22. The distribution curve in this case has little or no significance, as only one animal of the control series was held longer than 4 weeks.

No animals were inoculated in February, but, beginning in March, there was another period of increase which reached the maximum of 100 per cent on April 26. By June 7, the tendency to the occurrence of metastases was again extremely low. There was, however, a moderate but fairly sustained increase extending over the period from June 16 to August 15 and possibly on into late September.

In this connection, we should call attention to the fact that the series of September 25 and October 13 were the ones that gave such a high percentage of negative results on inoculation and that during July, August, and October many lesions showed a tendency to spontaneous resolution. This appears to be significant. At any rate, the two groups of animals inoculated during October showed a diminishing incidence of metastases which was followed by an even greater increase for November than was recorded for the same month of the preceding year. From the high point reached on November 10, the curve again dropped as it did in 1921.

It is not necessary to analyze the curves showing the distribution of metastases in the same way that we have analyzed that of incidence. It is obvious that, in the main, the three curves agree. There are however, a few apparent discrepancies which will be referred to presently.

If we condense the results recorded in Text-fig. 1, so as to base our calculations on larger groups of animals, using the month as the unit of time and including in a given group all of the animals inoculated during that month, the main effect is to smooth out minor irregularities in the curves and to emphasize the occurrence of two periods of maximum

malignancy—one in the spring and the other in the fall—as is clearly shown by that portion of the curve in Text-fig. 2 which gives the results for the year 1922.

It will be noted that this effect is not so apparent during 1921; this, in itself, is significant. Transplantation began in March and relatively high values were obtained in the first three series of animals that were inoculated (March, April, and May). During this time, there was no material change in the proportion of animals that developed metastases. In fact, the incidence of metastases diminished, but in spite of this the severity of the disease increased, as indicated by the progressive increase in the distribution of secondary tumors. In June, there was a reversal of the direction of the two sets of curves (incidence and distribution), and this was followed by a further readjustment in August which brought out the first concerted movement of the three curves. In this instance, the distribution curves remained at a high level during September, while the incidence curve had already assumed a downward direction, but, from this point onward, the changes became regular and of a clearly defined character, and the conditions shown for 1922 were repeated with slight variations in 1923.

DISCUSSION AND CONCLUSIONS.

The interpretation of the facts that have been brought out with regard to the operation of various factors that may have influenced the growth and malignancy of this tumor is comparatively simple. It is at once apparent that, in its final analysis, the problem that confronts us is that of the interaction between host and pathogenic agent. We have to consider, on the one hand, the effects of passage on the energy of cell growth, and the viability of the cells at the time of inoculation, and, on the other, the suitability of various classes of animals for the growth of these cells and the capacity of the animal to oppose their growth or to dispose of cells that are introduced, as these several conditions may be affected by such factors as age or breed and by the existing state of the animal organism.

The changes that occurred in the course of transplantation were conditioned on passage, and there is no doubt that the energy of cell growth was greatly increased by adaptation to passage. The indications are that this change was especially rapid during the first five or

six generations, but there is evidence to show that as the rate of growth and invasiveness of the tumor increased, the incitement to reaction was also increased so that a state of virtual equilibrium between these forces was soon established. This state of equilibrium might have been permanent had it been possible to maintain all the factors in the equation at a constant level, but as transplantation continued the equilibrium was disturbed by unavoidable variations in the experimental conditions so that the subsequent course of events became extremely irregular, depending upon whether the conditions that obtained at any given time were more favorable to the growth of the tumor or to the maintenance of an effective defense on the part of the animal.

It is apparent, therefore, as has been pointed out, that there were two conflicting tendencies in operation. One of these clearly was a tendency to an increase in the energy of cell growth which has ersisted in an effective form even to the present time; but the circumstances that favored opposition to the growth of the tumor are more difficult to define. We have considered the possibilities of variations in technique, chance inequalities of the material and of the animals used in different series of experiments, and the possible influence of the factors of time and the serial order of inoculation.

The results obtained cannot be explained as a manifestation of a simple equalization reaction in which each increase in the growth activity of the tumor called forth a corresponding increase in animal resistance. It is certain, however, that a reaction of this kind formed the basis for the changes that occurred. Irregularities in technique and chance variation in material and animals will account for some variation in results, but as one follows the succession of changes that occurred, he can hardly fail to be impressed by the suggestion of the operation of some obscure condition which tended to regulate the growth and malignancy of the tumor irrespective of the influence of other factors. This is most evident when we consider the results in serial order with respect to the time of inoculation. When viewed from this standpoint the entire series of changes assumes the form of an orderly succession of periods of increasing and diminishing malignancy with a distinct tendency to a seasonal distribution. This peculiar coincidence of effects is strongly suggestive of the action of some influence commonly referred to as meteorological.

It would appear, therefore, that the two main factors concerned in determining the results of transplantation were adaptation to passage and unavoidable variations in the conditions of season and weather that prevailed during the time these experiments were being carried out. It is possible to account for all of the variations in growth and malignancy that have been encountered in the course of routine transplantation as manifestations of the combined action of passage and meteorological conditions,—the one affecting the energy of cell growth and the other affecting animal economy. This aspect of the subject will be presented in detail in a subsequent paper.

SUMMARY.

The results of transplantation, as recorded in the first section of the paper, are analyzed from the standpoint of the influence of the factors of serial passage, the material used for inoculation, the age and breed of the animals, and the time or season at which the experiments were carried out.

The material used for the inoculation of different groups of animals appeared to have comparatively little effect on the ultimate results.

The character of the animals used was found to be a factor of more importance in that the tumor displayed definite peculiarities of growth and malignancy referable to age and to breed or color markings. Still, it seemed unlikely that any of these factors would account for the variations in growth and malignancy that had been observed.

After considering the various possibilities presented, it appeared that the principal factors concerned in determining the results of transplantation were adaptation to passage and variations in the conditions of season and weather that prevailed during the time the experiments were being carried out—the one affecting the energy of cell growth and the other affecting animal economy.

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THE INFLUENCE OF THE SUPRARENAL GLAND ON THE THYMUS.

II. Direct Evidence of Regeneration of the Involuted Thymus Following Double Suprarenalectomy in the Rat.

By HENRY L. JAFFE, M.D.

(*From the Laboratory Division of Montefiore Hospital, New York.*)

Plate 22.

(Received for publication, June 30, 1924.)

In this paper we present direct evidence of thymic regeneration occurring in the involuted glands of old rats following bilateral suprarenalectomy. The study is based on an examination of thirteen rats from which a portion of the right lobe of the thymus was removed as a control, followed by bilateral suprarenalectomy 13 to 17 days later. The histological appearance of the remaining portion of the thymus gland, removed at autopsy some weeks after suprarenalectomy, was compared with that of the piece removed at operation.

To our knowledge, no previous work exists in which attempts have been made to obtain unequivocal direct evidence of thymic regeneration following suprarenal ablation by examination of sections of the gland before and after suprarenalectomy.

In a previous communication (1) we have presented experimental evidence of the close interrelation between the thymus and suprarenal glands, which has been suspected for some time on the basis of clinical and pathological findings. Our results were based on a study of 66 doubly suprarenalectomized rats and 21 non-operated controls. In this work we found that following bilateral suprarenal ablation thymuses which should have showed advanced involution as estimated by the ages of the rats were hyperplastic, and on histological examination showed few signs of their previous regression. Enlargement of the thymus was observed to follow bilateral suprarenalectomy in these rats with such constancy that we concluded that practically every animal surviving double suprarenalectomy for 3 to 5 weeks would

show an enlarged gland. We assumed, therefore, from indirect but statistical data, that regeneration of the involuted thymuses must have taken place. The present study is a continuation of the work with the view of obtaining direct evidence of regeneration.

The previous experimental literature concerning thymic enlargement following suprarenal injury or ablation was critically reviewed in our last communication. We stated at that time, that no detailed studies existed, all previous reference to thymic enlargement following suprarenalectomy being reported incidentally.

EXPERIMENTAL.

The thymuses of the thirteen rats dealt with in this present study were examined 17 to 39 days after suprarenalectomy,[1] the animals dying or being sacrificed. The ages of the rats at autopsy varied from 282 to 427 days. The thymus in the normal rat begins to involute between the 80th and 90th days of age, and the process is progressive thereafter.

The experimental methods employed were the same as those used in the previous work. For the operative removal under ether of a fragment of thymus, the sternum was split down its middle to about the third rib, and the bone was retracted and the triangularis sterni muscle separated. The gland was exposed, and a small piece removed with as little trauma as possible and immediately fixed in 10 per cent formalin. The remaining portion of the gland was removed at autopsy and dissected from the surrounding fat and lymph nodes; it was then similarly fixed. All tissues were embedded in paraffin and stained with hematoxylin and eosin. The appended table presents the important data summarized from the protocols of the animals.

Examination of histological sections prepared from the fragments of the thymuses removed at operation shows that in all thirteen rats involution had already set in, being slight in one, moderate in four and well advanced in the remaining eight. Following bilateral suprarenalectomy the remaining portions of the thirteen thymuses showed histological evidences of secondary hyperplasia and regenera-

[1] All operations were performed under ether anesthesia.

tion, the regeneration being moderate in one animal and marked in the other twelve. Regeneration of the involuted thymuses took place irrespective of whether the animals died of chronic suprarenal insufficiency or were sacrificed. The presence of severe snuffles and extensive chronic pulmonary infections, or of marked loss of weight did not prevent regeneration in spite of the known fact that these influences ordinarily induce pathological involutions in this gland. Thus, it would seem that the stimulus for hypertrophy and hyperplasia of the thymus following bilateral suprarenal ablation is so marked that even the usual strong involutionary influences may be counteracted.

Experiments were carried out to establish whether or not partial thymectomy alone will induce compensatory secondary hyperplasia in an involuting thymus gland. Several rats were partially thymectomized, and the animals were sacrificed 2 weeks after operation. The histological appearance of the piece of thymus removed at operation was compared with that of the remaining portion of the gland removed at autopsy. We found practically no differences in the microscopical appearance of these glands before and after operation.

Histology.

The thymuses of the thirteen rats studied present, both grossly and microscopically, striking evidences of regeneration following bilateral suprarenalectomy. Rats over 1 year of age subjected to suprarenalectomy show at autopsy white and fleshy thymuses which are sometimes quite large. The histological differences between sections of the thymus before and after suprarenalectomy are frequently quite remarkable. Glands with advanced involution shown by the presence of irregular lobules which are reduced in size owing to a decrease in the small cortical cells, and in which the differentiation between the cortex and medulla has disappeared or has become obscure, and in which the connective tissue about the blood vessels has become prominent and hyalinized, undergo a rapid revivescence after suprarenalectomy. Such rejuvenated and regenerated glands resemble so closely histologically the growing active thymuses of normal young rats, 50 to 70 days of age, that were it not for the fact that the blood vessels show thickened walls with some increase of the connective tissue about them, it would be very difficult to distinguish microscopically between

TABLE I.

Rat No.	Sex.	Age at partial thymectomy.	Time between partial thymectomy and suprarenalectomy.	Time between suprarenalectomy and death.	Weight at death.	Thymus before suprarenalectomy.	Thymus after suprarenalectomy.	Major cause of death and remarks.
		days	*days*	*days*	*gm.*			
RR 6-1	F.	276	13	25	200	Slight involution.	Moderate regeneration.	Died. Suprarenal insufficiency; chronic pneumonia.
RR 6-2	"	276	13	31	148	Moderate "	Marked "	Sacrificed. Emaciation; chronic pneumonia.
RW 45-4	"	271	13	31	180	Advanced "	" "	Sacrificed.
RW 46-1	M.	303	13	31	250	Moderate "	Moderate "	" Chronic pneumonia.
RW 22-1	F.	272	14	39	148	Advanced "	Marked "	Died. Snuffles; suprarenal insufficiency; chronic pneumonia.
RW 23-2	M.	270	14	31	302	" "	" "	Sacrificed.
RW 24-4	"	270	14	18	300	" "	" "	" Chronic pneumonia.
RW 24-1	"	251	14	17	230	Moderate "	" "	"
RW 24-3	"	257	14	39	255	" "	" "	"
RW 19-1	F.	370	17	29	210	Advanced "	" "	Died. Acute pneumonia; chronic pneumonia.
RW 19-2	"	370	17	30	175	" "	" "	Sacrificed. Snuffles; moribund.
RW 19-3	"	370	17	30	235	" "	" "	" Chronic pneumonia.
RW 22-3	"	383	13	31	180	" "	" "	" " "

a regenerated thymus of an old rat and one of a normal rat at about puberty. These regenerated glands show large bulging, closely apposed lobules separated by thin fibrous tissue septa and these lobules stand in marked contrast to the small and irregular lobules present in the same gland before suprarenalectomy. The regenerated lobules are differentiated into distinct cortical and medullary zones. The cortex is thickened and packed with closely aggregated small thymic cells. The medulla shows the most striking change. It rapidly reappears as large, active centers, composed largely of hypertrophied reticular cells. Another striking feature, in regard to the medulla, is that many lobules in a regenerated gland show, in addition to a large medullary mass, as many as two or three smaller medullary areas, which are probably additional growth centers (Figs. 1 and 2). At the present time we are not in a position to express an opinion as to possible changes following suprarenalectomy in the Hassall's corpuscles, or in the epithelial ducts so frequently seen in the rat thymus.

DISCUSSION.

We have now, by utilizing suprarenalectomy, a method of studying rapid regeneration of the involuted thymus that is free of the objectionable features associated with the generally used but highly unnatural and unphysiological methods of roentgenization and inanition. By the use of direct experimental methods, we have shown that removal of the suprarenal glands in old rats will induce regeneration of involuted thymuses, thus confirming our previous work on the rat and the observations of Marine, Manley, and Baumann on the rabbit (2). Regeneration of involuted thymuses following bilateral suprarenalectomy took place irrespective of whether the animals died of chronic suprarenal insufficiency or were sacrificed. The presence of severe snuffles and extensive pulmonary infections, or marked loss of weight did not prevent regeneration.

While our thymuses were examined when regeneration was already well established or complete, that is 17 to 39 days after suprarenalectomy, it is our belief from this and previous studies that regeneration begins early, within 24 hours after suprarenalectomy, and progresses rapidly, being completed in the rat in most cases within

2 weeks. We are also of the belief that the reticular cells are probably the first to respond to the growth-stimulating influence exerted on the thymus following suprarenalectomy, the medulla regenerating before the cortical regeneration is complete.

The mechanism involved in the regeneration of the thymus which follows suprarenalectomy is still not understood, and while thymic hyperplasia may be one manifestation of the generalized lymphoid hyperplasia that follows sublethal but sufficient suprarenal injury, nevertheless, the fact should not be lost sight of that thymic enlargement may represent a specific reaction of this organ to suprarenal injury.

The finding is of significance since it is recognized that the suprarenal glands play an important etiological rôle in two of the three most common diseases presenting thymus enlargement among the major pathological findings at autopsy; these are Addison's disease and status lymphaticus. While anatomical studies have not disclosed any constant lesions in the suprarenals in Graves' disease, evidence is being accumulated which would indicate that functionally these glands also play an important if not primary rôle in the production of the clinical syndrome of Graves' disease (3). It is now generally accepted that both in Addison's and in Graves' disease regeneration of the involuted thymus occurs, which may take place even in the presence of profound emaciation or chronic infection. We are of the belief that the large thymus which occurs in status lymphaticus, and the regeneration which occurs in Addison's and Graves' disease are brought about by the same disturbances in glandular interrelations which bring about regeneration of the thymus in the experimental animal after suprarenalectomy.

CONCLUSIONS.

1. Regeneration of involuted thymuses of old rats follows double suprarenalectomy in this species with great constancy.

2. Partial thymectomy alone does not induce compensatory secondary hyperplasia in involuting glands.

3. Regeneration of the thymus following suprarenalectomy would appear to be completed in most rats in about 2 weeks; and it probably begins in the medulla.

I wish to express my sincere appreciation of the suggestive and stimulating criticism of Dr. Marine which has made this work possible.

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3. Marine, D., and Baumann, E. J., Influence of glands with internal secretions on the respiratory exchange. V. Further observations on the effect of suprarenal insufficiency (by removal) in thyroidectomized rabbits, *J. Metabol. Research*, 1922, i, 777.

EXPLANATION OF PLATE 22.

FIG. 1. A portion of the thymus of RW 22-3 before suprarenalectomy. The animal was 383 days of age, and section shows well established involution. Hematoxylin and eosin. × 60.

FIG. 2. The thymus of rat RW 22-3 31 days after suprarenalectomy. Section shows marked regeneration and secondary hyperplasia. Hematoxylin and eosin. × 60.

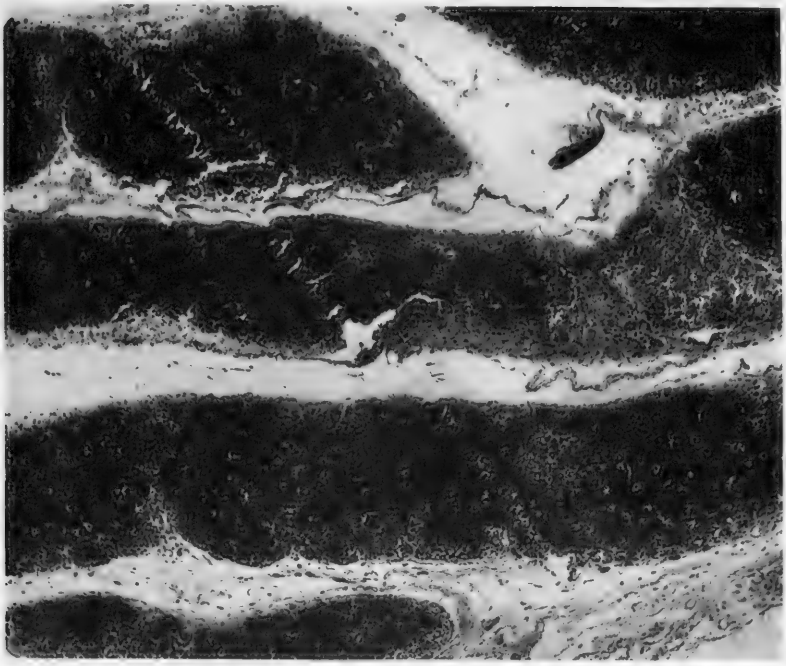

FIG. 1.

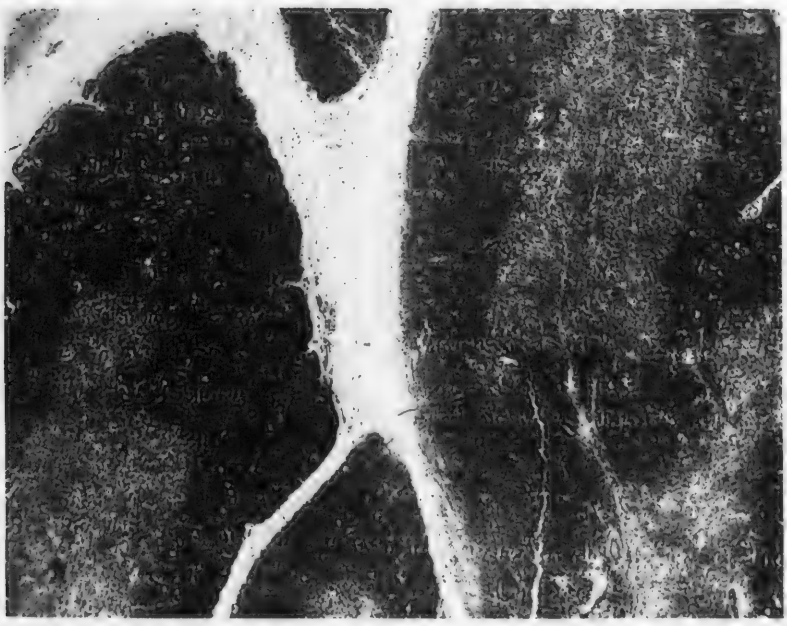

FIG. 2.

(Jaffe: Influence of suprarenal gland on thymus. II.)

THE EFFECT OF HEAT ON FLAGELLAR AND SOMATIC AGGLUTINATION.

By MARION L. ORCUTT.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In 1904, Beyer and Reagh[1] differentiated between flagellar and somatic agglutinins by means of heat. They worked with a motile and a non-motile culture of hog-cholera bacilli from different sources. They found that a temperature of 70°C. injured the flagellar agglutinating substance and the somatic agglutinins but did not injure the flagellar agglutinins nor the somatic agglutinating substance. Furthermore they reported that a motile culture heated to 70°C., although it no longer gave a flagellar agglutination reaction, still would cause the production of flagellar agglutinins in the animal body.

These experiments have been repeated in the course of the work here to be reported, but this time the two forms of the one strain hog-cholera Md. were used. This hog-cholera Md. culture has been under cultivation since 1898. It was originally a motile strain but in 1923 a non-motile form was isolated from it and this mutant has been kept as a separate strain since that time. The separation of the flagella from the bodies of the bacilli and the use of the separated flagella as a separate antigenic substance has been reported in a previous paper.[2] In the present experiments the non-motile mutant of the Md. culture was used for the somatic agglutinogen, the separated flagella for the flagellar antigen, and the sera produced by these two forms supplied the flagellar and somatic agglutinins. In general the results agree with those of Beyer and Reagh.

When the serum produced by the separated flagella was heated at 70°C. for 20 minutes (Table I) it still agglutinated the flagella, but the somatic serum pro-

[1] Beyer, H. G., and Reagh, A. L., *J. Med. Research*, 1904, xii, 313.

[2] Orcutt, M. L., *J. Exp. Med.*, 1924, xl, 43.

TABLE I.

Flagellar suspension.	Serum dilutions. 1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Control.	Remarks.
Flagellar serum (Rabbit 6-40), unheated.											
Unheated.	+++*	++	++	+	+	−	−	−		−	
Heated, 70°C.	+ (fine)	−	−	−	−					−	No fluffy flagellar reaction.
Flagellar serum (Rabbit 6-40), heated, 70°C., 20 min.											
Unheated.	+++	++	+	+	−	−				−	
Heated, 70°C.	−	−	−							−	

*+ = visible clumping; ++ and +++ = stronger reactions; no standard for complete reaction because original suspension is clear.

TABLE II.

Non-motile culture.	Serum dilutions. 1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Control.
Non-motile culture serum (Rabbit 5-84), unheated.										
Unheated.	C.	C.	C.	C.	C.	++++	++	+	−	−
Heated, 20 min., 70°C.	"	"	"	++++	++++	+++	++	+	−	−
" 20 " 75° "	"	"	+++	+++	++	++	+	±	−	−
" 20 " 100° "	"	"	C.	++++	+++	++	+	+		−
" 20 " 120° " (autoclave).	"	"	"	++++	+++	++	++	+		−
Non-motile culture serum (Rabbit 5-84), heated, 70°C., 20 min.										
Unheated.	+++	++	+	±	−	−	−	−		−
Non-motile culture serum (Rabbit 5-84), heated, 75°C., 20 min.										
Unheated.	−	−	−	−	−	−	−	−		−

duced by the non-motile mutant when heated at 70°C. gave a considerably reduced reaction with that culture (Table II). Again, the flagella heated at 70°C. for 20 minutes and then tested with the unheated serum gave no agglutination reaction (Table I), but the non-motile mutant heated at 70°C. and tested with

the unheated somatic serum gave a final titer equal to that of the unheated culture. In these tests 70°C. did not give as complete a destruction of somatic agglutinin as the results of Beyer and Reagh indicated, although it caused a considerable lowering of the reaction. For instance, in the results of Beyer and Reagh the serum produced by the non-motile strain heated at 70°C. gave no reaction with the unheated bacilli, while in the corresponding experiment repeated at this time the serum produced by the non-motile mutant heated at 70°C. gave a strong reaction at 1:20 and a trace of clumping at 1:160 in contrast with the result with the unheated serum which gave a complete agglutination at 1:20 and a slight reaction at 1:2,560. If a temperature of 75°C. was used the agglutinating power of this serum was completely destroyed (Table II), but this temperature altered the flagellar serum so that a zone of inhibited agglutination occurred in the lower dilutions of 1:20 to 1:40 and the whole reaction took place more slowly than with the unheated serum. This temperature of 75°C., however, had little effect on the agglutinating substance of the non-motile bacilli. In fact, a temperature of 100°C. or even 120°C. did not destroy their agglutinating substance. Tests were carried out with cultures of the non-motile mutant heated in a water bath for 20 minutes at 70°, 75°, 80°, 85°, 95°, 100°C., and heated in the autoclave at 120°C., and an unheated culture as control. They all gave a similar agglutination reaction in the unheated somatic serum. The unheated culture gave a greater degree of completeness but the titer limit was the same for both heated and unheated cultures.

The second experiment by Beyer and Reagh was the production of a flagellar antiserum with a motile culture heated at 70°C. This experiment was repeated with a suspension of separated flagella heated at 70°C. Such a suspension no longer gave an agglutination reaction. However, on injection into a rabbit it gave rise to a serum containing flagellar agglutinins just as did the unheated flagellar suspension.

A microscopic examination of the heated and unheated cultures revealed certain differences.

A preparation of the unheated flagella stained with a flagella stain showed many wavy flagella, some in more or less of a network and some scattered. In a preparation of the same suspension heated at 70°C. and stained with the flagella stain none could be distinguished. A preparation of the unheated motile bacilli showed the organisms with long wavy flagella and this same culture heated at 70°C. and stained with the flagella stain showed the bacilli as distinctly as in the preparation of the unheated culture but the flagella were no longer seen. Sometimes granular lines appeared between groups of bacilli suggesting flagella but quite different in appearance from the flagella of the unheated culture. On the other hand, the non-motile mutant unheated and heated up to 100°C. in a water

bath and 120°C. in the autoclave when stained with flagella stain or with carbol-fuchsin, methylene blue or Stirling's gentian violet showed a similar appearance. The outline of the bacilli was as definite in the preparation of the heated culture as in that of the unheated culture, but sometimes the heated bacilli seemed a little more faintly stained.

These observations indicate that the heat destroys the structure of the flagella but does not cause the disintegration of the bodies of the bacilli. This helps to explain why the heated flagella gave no visible reaction while the heated bacilli, which still kept their form intact, continued to react visibly.

Another experiment was made by absorbing the flagellar serum with heated flagella and the heated motile culture and then centrifuging and retesting with fresh unheated flagellar suspension and motile bacilli. This experiment was performed three times and the last time a very heavy suspension of flagella and a heavy growth of the motile bacillus was used for the absorption. In every case the results were the same. The serum after contact with the heated flagella or the heated motile culture still gave a strong reaction when the fresh unheated culture or flagella were added. This result indicated that the disintegrated flagella not only failed to become clumped but did not even combine with the agglutinins. However, since the heated flagellar suspension produced flagellar agglutinins in the animal the antigenic nature of the flagella was not destroyed by the heat although their form, their ability to clump, and their ability to absorb agglutinins were destroyed. The reactions of each of the four different substances to heat have been summarized in Table III.

Eisenberg and Volk[3] stated that in the case of typhoid bacilli, heat above 60°C. injured the agglutinating ability of the bacilli but did not destroy their power to absorb agglutinins, and they explained this reaction by the presence of two factors in the agglutinating substance, a thermolabile clumping factor and a thermostable binding factor. At the time of their work no distinction had been made between flagellar and somatic agglutination. From previous work[4, 2] we know that a motile culture contains two antigenic substances, a flagellar antigen and a somatic antigen, and the present results show

[3] Eisenberg, P., and Volk, R., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 155.

[4] Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, x, 89.

that the flagella are destroyed by heat so that they no longer give an agglutination reaction nor do they absorb agglutinins, while the bodies of the bacilli are not destroyed by heat and they still react with agglutinins.

The results of the present observations with the non-motile mutant and the separate flagella show a general agreement with the work of Beyer and Reagh on the differentiation of flagellar and somatic agglutinins by heat. The results indicate that heat destroys the structure

TABLE III.

Effect of Heat on Hog-Cholera Md. Flagellar and Somatic Antigen and Agglutinins.

Flagellar antigen.	+	70°C.	= Destruction of form and of ability to clump and to absorb agglutinins, but no destruction of the power to generate or stimulate agglutinin production.
Somatic "	+	70°C. or heat up to 120° C.	= Little or no destruction of form or of ability to absorb agglutinins and form clumps.
Flagellar agglutinins.	+	70°C.	= Little or no destruction of agglutinating ability.
" "	+	75°C.	= Alteration, with occurrence of inhibition zone in lower dilutions.
Somatic "	+	70°C.	= Considerable injury to agglutinating ability.
" "	+	75°C.	= Destruction of agglutinating ability.

of the flagella but not that of the bodies of the bacilli, and finally that flagella or motile bacilli heated at 70°C. do not absorb flagellar agglutinins.

CONCLUSIONS.

1. Heat at 70°C. destroys the form of the flagella and their ability to combine with flagellar agglutinins but it does not destroy their antigenic nature since they can still generate flagellar agglutinins in the animal body.

2. Heat at 70°C. and even at 120°C. in the autoclave does not destroy the forms of the bacilli themselves nor their ability to become agglutinated and to absorb agglutinins.

3. Somatic agglutinins are destroyed to a considerable extent by heat at 70°C. and completely destroyed by heat at 75°C.

4. Heat at 70°C. causes little or no destruction of flagellar agglutinins but a temperature of 75°C. changes the agglutinins so that they react more slowly and produce a slightly lower reaction with a zone of inhibition in the stronger dilutions.

THE PENETRATION OF BACTERIA THROUGH CAPILLARY SPACES.

IV. A Kinetic Mechanism in Interfaces.

By STUART MUDD, M.D., and EMILY B. H. MUDD.

(From the Mount Desert Island Biological Laboratory, Salisbury Cove, and the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 23 and 24.

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Before undertaking a more direct study of the physical-chemical factors involved in the penetration of bacteria through the epithelia of the animal body, and as an essential guide in such an investigation, it has seemed desirable to study mechanisms capable of transporting bacteria in and through simpler systems. Certain critical relations of bacterial dimensions and motility to pore size of filters,[1] effects of electric charge and electroendosmotic streaming,[2,3] and bacterial chemotropisms[4] have already been considered. The following notes describe a kinetic mechanism dependent primarily upon interfacial surface tension forces in the boundary between two wholly or partly immiscible fluids. The question whether or not such a mechanism plays a part in actual processes of infection will have to wait upon further study.

TECHNIQUE.

The preparations studied are two-phase liquid films with bacteria suspended, usually in the aqueous phase. Films and bacteria are made visible with a dark-ground illuminator. A small drop of one liquid, usually distilled water, sea water, or Ringer-Tyrode solution, is placed upon a clean slide on the microscope stage. Bacteria, scraped with a platinum loop from an agar slant, are suspended in this

[1] Mudd, S., *J. Bact.*, 1923, viii, 459.

[2] Mudd, S., *Am. J. Physiol.*, 1922–23, lxiii, 429.

[3] Mudd, S., and Mudd, E. B. H., *J. Bact.*, 1924, ix, 151.

[4] Warren, S., and Mudd, S., *J. Bact.*, 1924, ix, 143.

drop of liquid. A clean cover-slip is laid over the suspension.[5] It is desirable that the drop be large enough to give a good film beneath the cover-slip but not so large as to cover the entire under surface of the cover-slip. A drop of the organic liquid—oil, hydrocarbon, alcohol—is caused to run under the cover-slip from one side, meeting the aqueous phase and with it forming a two-phase film between cover-slip and slide. In some organic liquids the bacteria can be suspended first and the water then added. In general, however, this procedure is less satisfactory, because from most organic films thus examined ordinary bacteria are rapidly absorbed on the glass. An alternative method is to place drops of the two liquids to be used at two points on the slide, suspend the bacteria in one of them, and carefully lay a cover-slip over both.

The preparation may then be studied with the dark-field and any desired set of lenses, including the oil immersion. If the organic liquid is too volatile or if it is desired to preserve the preparation for study on subsequent days, the edges of the cover-slip are sealed with a high melting point paraffin. Grübler's 68–72°C. paraffin has been used in our experiments with satisfaction. Paraffin of lower melting point (52°C.) and vaseline have been found to melt in the radiation from the Bausch and Lomb substage illuminator used.

If the preparation is too brilliant for convenient study, the light can, of course, be cut down as desired with a slide-wire rheostat.

OBSERVATIONS.

The boundary line or interface between organic and aqueous phases appears as an intensely luminous line or band, usually with a number of alternate dark and bright interference fringes parallel to, and, typically, on the water side of the interface. The two phases are dark grey or brown or black, according to their optical purity and the illumination. The bacteria appear as golden motes in the interface and water phase and in the organic liquid usually as more silvery particles adherent to the glass. Air bubbles are ordinarily recognizable by their surrounding border of even more dazzling brilliancy than the liquid-liquid interface.

[5] The slides and cover-slips should first be washed with soap and water, then soaked or boiled in dichromate-sulfuric acid cleaning solution, washed, soaked in alcohol, and finally washed in a jet of steam. After the soap and water washing they are handled only with metal forceps. The slides may be kept until used in a clean dry staining dish and the cover-slips in a closed container, preferably on clean filter or lens paper. If necessary they may be carefully wiped with clean lens paper before using.

Observation of such preparations has resolved itself into a study of mechanisms for the concentration of bacteria in the liquid-liquid interface, their transportation, often for considerable distances along the interface, and either their eventual escape from the interface or their agglomeration into solid or semisolid bands in the phase boundary lines. The second phenomenon mentioned, namely movement of bacteria along the interface between organic liquid and water, is more relevant to the general subject of these studies, and will, therefore, be described before considering the mechanism of concentration of bacteria in the interface.

Interfacial Kinetic Mechanism.—The bacteria in the interface were subject to forces which made their behavior distinctively different from that in either water or organic phase. The most characteristic phenomenon observed was a sliding or streaming along the interface. Such movements occurred in films of every composition studied, whether interfacial tension was low or high, and whether the water and organic fluid were miscible or immiscible. The bacteria slid along the bright interfacial line so slowly that their motion could scarcely be detected, or so swiftly that with the high powers of the microscope it could be followed only with difficulty. Individuals and masses were carried along the interface as though in a groove, following the straight or intricately folded boundary line often through several low power fields (each field of diameter 1.24 mm.)

The streaming of microorganisms along the boundary often carried them to a certain point at the margin of the cover-slip. In other places movement was from both directions to a zone in the interface in which the bacteria were crowded together to form a practically solid membrane; subsequently streaming away from this zone in both directions might occur.

Such movements were for the most part independent of movement in the contiguous phases. Often a drift of the bacteria in the water and organic liquid would occur as a result of evaporation or other cause, while the bacteria in the interface a few microns away were streaming in the opposite direction. Or the bacteria in the interface would slide by bacteria in the phases which were not suffering any displacement; or those in the liquids would drift by stationary bacteria in the boundary line. Certain correlations, however, did occur. For

instance, if large bacteria or, especially, bacterial masses were sweeping along the interface, the bacteria nearby were often carried along somewhat less rapidly in the same direction, owing doubtless to the internal friction of the liquid. And when strong currents were produced in the film, for instance by mechanical pressure on the coverslip, these often affected bacteria both in the phases themselves and, in a lesser degree, in the interface.

Certain characteristics of these interfacial displacements could be correlated with the properties of the fluids used. For instance, when interfacial tension was high (as in the case of cyclohexane, tension of water-organic liquid interface (T_{ow}) = 60.6 dynes per cm.) the movements of bacteria in the interface were often jerky and spasmodic, irresistably conveying the impression of particles fixed in a taut elastic medium which yielded locally at times. When interfacial tension was low and the two liquids were to a considerable degree miscible, as in the case of the alcohols (Table I), motion in the interface was more a streaming, often vigorous but always smooth.

Movements of bacteria along the interface occurred, as has been said, in films of all compositions studied. The organic liquids used in this study have been the following neutral and essential oils, hydrocarbons, and alcohols: sperm, sheep's foot, rape-seed, olive and cedar oils, eucalyptol, oils of lavender, thyme, sweet birch, sassafras, and peanut; liquid albolene, liquid petrolatum Squibb, cyclohexane; cyclohexanol, isobutylic and isoamylic alcohols. Caprylic and oleic acids, carbon tetrachloride and ortho-toluidine have shown similar behavior. Distilled water, sea water, and Ringer-Tyrode solution have been used as aqueous phase.

The bacteria used have been the Gram-negative *Bacterium coli communis*, *Vibrio percolans*, and *Erythrobacillus prodigiosus*, and the Gram-positive *Bacillus subtilis* and *Bacillus megatherium*. No significant differences between them as regards the phenomena under discussion have been observed. Under the circumstances of the present experiments the bacteria were non-motile or virtually so. The behavior of bacteria in an actively motile condition and of members of the acid-fast group will be discussed in the following paper.

Similar gliding movements of bacteria along water-air interfaces have also been observed. These interfaces are so dazzlingly bright, however, that they are difficult to study.

Other Movements.—Other movements in the system may now briefly be described. Irregular drifts and currents in the water and organic liquids due to evaporation from the margin of the preparation, pressure, unequal expansion with heating and what not are often much in evidence. When the edge of the cover-slip is sealed with paraffin, these irregular currents in the phases are minimized but usually not wholly eliminated, particularly if air bubbles are contained beneath the cover.

TABLE I.

Interfacial Tension and Solubility of Some of the Organic Liquids Used.

Substance.	Solubility in water per 100 cc.	Interfacial tension (T_{ow}) against water per cm.
	gm.	*dynes*
Cyclohexane.	Insoluble.*	60.60†
Cyclohexanol.	5.67 (11°C.)‡	3.92†
Carbon tetrachloride.	0.80 (20°C.)‡ p. 239.	52.63†
Caprylic acid.	0.25‖	8.217†
Isobutylic alcohol.	Gm. alcohol per 100 gm. at 20°C. ‡ p. 164 Water layer 9 Alcohol layer 84	1.76†
Isoamylic "	Gm. alcohol per 100 gm. at 22°C.§ Water layer 2.61 Alcohol layer 97.36	4.42†
Liquid petrolatum (Squibb).		55.32**

* Hodgman, C. D., and Lange, N. A., Handbook of physics and chemistry, Cleveland, 1922, 180.

† Harkins, W. D., Brown, F. E., and Davies, E. C. H., *J. Am. Chem. Soc.*, 1917, xxxix, 356.

‡ Seidell, A., Solubilities of inorganic and organic compounds, 2nd edition, enlarged and revised, New York, 1919, 280.

§ Seidell, A., Solubilities of inorganic and organic substances, 2nd edition, New York and London, 1911, 11.

‖ Thorpe, E., A dictionary of applied chemistry, London, New York, Bombay, and Calcutta, 1912, 723.

** Harkins, W. D., and Feldman, A., *J. Am. Chem. Soc.*, 1922, xliv, 2665.

The bacteria free in the water undergo active brownian movement. Upon preparation of the film relatively few of the bacteria usually are stuck to the glass. As time goes on, more and more adhere. In the organic liquid such few bacteria as are suspended do or do not undergo brownian movement, according to the viscosity of the medium. In a large majority of the preparations studied, however, the bacteria, if they become suspended in the organic liquid at all very soon adhere to the glass separately or in clumps. A number of times groups of bacteria, lately come into the organic liquid from the water phase, have been observed to clump more closely together, as though drawn by an invisible elastic membrane, and to stick to the glass.

The bacteria in the interface also usually undergo brownian movement unless clumped or stuck to the glass. In some thirty-three experiments in which this point was studied, twenty-three record that brownian movement occurred in the interface. Ten show that it could not be observed there. The movement, however, was usually less free than in the water. The amplitude of the oscillations was less than in the water and the positions of the axes of the bacteria underwent less change than in the water.

As a result of the drifts and currents described, the organic liquid-water boundary line moves about considerably. As it catches, so to speak, on bacterial masses stuck to the glass, the interface sometimes tears the bacteria loose from the glass and sweeps them ahead and on the aqueous side of the advancing boundary line; more often perhaps the interface is itself increased in area and drawn out into rounded projection (interfacial tension high (see Fig. 4)) or finger-like or even very pointed peninsulas (interfacial tension low). The pointed projections are naturally most common in films with very low interfacial tension, the alcohols against water, for instance (see Fig. 2). If the peninsulas were drawn out too long they either retracted, often leaving bacteria individually or in clumps in the organic liquid, or they broke off, leaving a droplet of water containing bacteria in the organic phase (see Fig. 3).

The freedom of brownian movement of bacteria in the organic liquid-water interfaces was less with cyclohexane than with its corresponding alcohol. No brownian movement was noted in four of six experiments with cyclohexane-water interfaces. In the cyclohexanol-

water boundary line very little restraint of the brownian movement of the suspended microorganisms was observed.

The freedom of brownian movement in the interfaces and escape from the interface show interesting correlation. If we designate the fact that bacteria in any given experiment were observed to escape from the interface as + and that none were observed to escape as −, and similarly that brownian movement was observed in the interface as + and that none could be detected as−, in the records of twenty-four experiments, we find the two observations alike in sign in twenty-one and different in sign in only three experiments.

A number of times with cyclohexane-water a long bacterium was observed with one end in the interface and the rest in the water. The part of the microorganism in the water danced about the end in the interface as if about a pivot fixed in a groove, as the whole organism slid along the interface, slowly following its hollows and crests like a minute traveling flail.

Interfacial Trapping Mechanism.—It is characteristic of the preparations under discussion, with the exception of the alcohols and other organic liquids the surface tension of which against water is very low, that bacteria accumulate in the interface in higher concentration than in the liquid phases proper. This accumulation tends to occur more rapidly and to a greater degree in boundaries between liquids of which the interfacial tension is high than in those whose interfacial tension is lower. An hour after preparation of a cyclohexane-water film, for instance, many regions of the interface are usually immobilized by solid or virtually solid membranes of adsorbed bacteria. After a few hours, strong currents may often be caused in such a preparation by pressure on the cover-slip without displacing the interfaces fixed by adsorbed bacteria. With cyclohexanol-water films, on the other hand, the interfaces remain mobile, and streaming of bacteria along them may be seen after days.

The bacteria may reach the interface by brownian movement or with the aid of currents. More effective, however, are movements of the boundary line itself, causing it to encroach on the water phase and overtake the bacterial individuals and masses therein. Careful and patient scrutiny with the oil immersion objective has not shown attraction of bacteria into the interface through microscopically

visible distances. Such perceptible attraction was not to have been expected since the range of action of molecular attraction is of the order of magnitude of 10^{-8} cm. and thus is not capable of microscopic resolution.[6]

When once in the interface, however, the bacteria are trapped there and do not escape, unless work is done upon them. To remain in the interface is so much the rule for suspended bacteria and to leave it the exception that it is perhaps best to enumerate the means by which escape of bacteria from the boundary line has been effected. It is to be remembered, however, that bacteria adsorbed on the glass in the water were engulfed by the organic liquid as it encroached on the aqueous phase in practically all experiments.

Escape from Interface into Organic Liquid.—In thirty experiments there is definite record that suspended bacteria were not seen to cross from the water phase or interface into the organic liquid. In a number of experiments this point was not recorded. In twenty-one experiments bacteria were observed to cross into the organic phase under the special circumstances detailed below.

1. Bacteria, especially when accumulated in masses in the interface, often lagged behind it into the organic liquid as the boundary line gently advanced on the water. This occurred usually when there was reason to believe, because of the composition of the film or the scalloped contour of the interface, that interfacial tension was low. It was noted in eight out of twelve experiments with littoral sea water, which is known to contain surface-active substances, and may have occurred, but was not specially recorded in the others. This lagging behind the interface was, otherwise, noted especially with the alcohols. The following instances are recorded: sea water against oils of lavender, sweet birch and peanut, olive oil, sheep's foot oil, eucalyptol, albolene and *o*-toluidine; distilled water against eucalyptol, isoamylic alcohol, cyclohexanol (three experiments in five), cyclohexane (one in six experiments), C Cl_4; Ringer-Tyrode solution against cyclohexanol.

2. When a peninsula of water containing bacteria retracted, bacteria were often left behind in the organic liquid in a little water vacuole or stuck to the glass, and sometimes freely suspended in the oil (see Fig. 3). Sperm oil-distilled water; oil of sassafras-distilled water; C Cl_4-distilled water.

3. When suspended clumps of bacteria were overtaken by a rapidly advancing interface they sometimes broke through into the oil: sea water against olive oil and oils of lavender and sweet birch; distilled water-sperm oil.

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[6] Lewis, W. C. McC., A system of physical chemistry, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1918, i, 10.

4. When bacteria were strongly swept along the interface into a certain region until masses were formed and these became too large, certain bacteria were forced out of the interface into the organic phase; eucalyptol-sea water, peanut oil-sea water; cyclohexanol-distilled water; cyclohexane-distilled water.

5. Occasionally bacteria in active brownian movement in the interface danced out into the organic phase: eucalyptol-sea water; cyclohexanol-distilled water; C Cl_4-distilled water.

6. When bacteria were rounding a sharp curve in the boundary line at high speed, they were thought in several instances to be flung out by centrifugal force into the oil; sperm oil-distilled water (two experiments).

Escape from Interface into Water.—There are many circumstances also in which bacteria escape from the interface into the aqueous phase. The following examples may be cited.

1. When bacteria had been carried into one region of the interface until large numbers accumulated there, some were forced out of the interface into the water: sea water-eucalyptol.

2. Bacteria in brownian movement in the interface danced out into the water: sea water-oil of lavender, sea water-oil of thyme, distilled water-cyclohexanol (two experiments), distilled water-caprylic acid.

3. When bacteria were swept at high speed down the interface, they occasionally struck masses of fixed bacteria with sufficient force to be bounced out of the interface into the water: distilled water-cyclohexanol.

4. Bacteria rounded a sharp bend in the interface so fast as to be thrown out into the water: sperm oil-distilled water; cyclohexanol-distilled water.

5. When an oil phase not in contact with water ran against the aqueous phase, some bacteria were knocked out of the air-water interface into the water: eucalyptol-distilled water.

In examining the accompanying plates it should be remembered that the films have appreciable depth, and that the organic liquid, water, and probably the interface have different indices of refraction. It is not possible, therefore, to bring all parts of the preparation into a single focal plane. A wealth of detail and the fine definition which can only be brought out by constant manipulation of the fine adjustment are therefore necessarily lost in photography.

The preparations taken through low power lenses—× 165—were sealed and allowed to stand for a few hours, after which they were still enough to photograph.

For high power views, Mr. Louis Schmidt took moving picture films and enlarged individual pictures. The film from which Fig. 5 is taken very successfully showed the streaming movements in the interface and the brownian movement of bacteria in the water phase. In the enlargement, bacteria the excursions of which in the focal plane were large, *i.e.* those moving in the interface and several in the water, appear as bright streaks rather than points. The bright specks visible in the interface at *p* and *q*, Fig. 5, were bacteria stuck to the glass.

Interpretation.

It is clear from inspection of the bacteria in the aqueous-organic liquid interfaces of 60 odd experiments that work must be expended upon the bacteria to remove them from the interface when once there. It seems safe to deduce from the Gibbs-Thomson principle therefore that the presence of the bacteria in the interface lowers the free surface energy of the interface. This conclusion harmonizes with the observation that the escape of the bacteria from the interface seems to be more difficult when the interfacial tension is high than when it is low, *e.g.* bacteria are more effectively trapped in cyclohexane-water than in cyclohexanol-water interface.

The movement of bacteria along the interface has occurred in films of all composition studied, including those in which the water and organic liquid are not appreciably miscible, *e.g.* cyclohexane, mineral oil. This streaming in the interface occurs whether or not there is any drift of the particles in the contiguous water and organic phases and often in opposite direction to displacements of the bacteria in the phases if such are occurring. Similarly drift of the water and oil may occur past stationary bacteria in interfaces. Moreover, displacements of bacteria in interfaces of high surface tension are often distinguished by their jerky, tense character. From these facts we may conclude that local changes in surface tension caused, for instance, by local concentration of surface-active substances or uneven heating of the interface, constitute one driving mechanism for the bacteria in their movement along the interface.

On the other hand, streaming of the bacteria is quite as vigorous in interfaces between fluids whose interfacial tension is low but whose mutual solubility is considerable (*e.g.* isoamylic, isobutylic alcohols, and cyclohexanol against water) as in interfaces between liquids of high interfacial energy which are immiscible. In such preparations of miscible liquids, moreover, microscopic whirlpools and currents in the phases near the interface are likely to occur. We conclude from these facts that minute currents along and across the interface, due to mixing of the two liquids, constitute a driving mechanism for the bacteria in addition to, although not wholly separable from, changes in surface tension.

A more detailed analysis of the phenomena here recorded in terms of the interfacial surface tension relations will be given in the paper immediately following.

DISCUSSION.

Quincke[7] in 1879 observed a drop of neutral oil containing a little fatty acid and suspended in alkali solution. The fatty acid and alkali met at the surface of the oil drop, formed soap and this, lowering surface tension locally, was spread by the tension of the interface over the surface of the drop. Some of the contiguous water was drawn along by the spreading soap film and other water moved into its place, thus giving rise to currents in the solution. Loeb and others have applied such observations to the explanation of certain intracellular phenomena.[8,9] The movements of bacteria along the interfaces described in the present communication are evidently similar phenomena to those of spreading described by Quincke. Movements of bacteria along lipoid-water interfaces have been predicted by Wright on theoretical grounds and urged

[7] Quincke, G., *Arch. ges. Physiol.*, 1879, xix, 129; *Sitzungsber. berl. Akad. Wissensch.*, 1888, 791.

[8] Loeb, J., The dynamics of living matter, New York and London, 1906, 55, 65.

[9] Ewart, A. J., On the physics and physiology of protoplasmic streaming in plants, Oxford, 1903, 112.

by him as of capital importance in the initiation of infection.[10] We should prefer to reserve judgment.

SUMMARY.

The dark-field microscope may be used to observe directly the characteristics of composite films. The liquid phases, one or both of them containing suspended solid particles as test objects (in these experiments bacteria were used), are spread between slide and coverglass and examined with any desired lenses. The liquid-liquid interfaces appear as bright lines and the solid particles as shining motes.

An interfacial kinetic mechanism has been observed in films of all composition studied. The bacteria are transported along the phase boundary lines in a striking and characteristic manner and quite independently of movements in the adjoining organic or aqueous phases. These movements in the interface are interpreted as essentially due, according to the composition of the films, to local inequalities in interfacial surface tension, or to minute currents from mixing of the two phases across and along their boundary line, or to both forces acting together.

The bacteria (non-motile in these experiments) reached the interface by brownian movement or currents or shifts in the position of the boundary line. Once in the interface they tended to remain, and accumulated there, in instances where the liquid-liquid interfacial tension was high at least, in higher concentration than in the contiguous phases. Bacteria could, however, escape from the interface in a variety of ways detailed above.

With liquids which differ markedly in interfacial tension and miscibility with water, these properties may be correlated with the characteristics of the preparation. With cyclohexane-water films, for instance, (immiscible, interfacial tension high), the boundary was less readily drawn out into projections, the interfacial trapping mechanism was more efficient, and brownian movement of bacteria

[10] Wright, J., *New York Med. J.*, 1906, lxxxiii, 17; lxxxiii, 117; lxxxiv, 1161; 1907, lxxxv, 289, 435, 537, 627, 769; 1910, xcii 749; 1911, xciii, 257, 354, 662, 764.

in the interface was less free than with cyclohexanol-water films (miscible, interfacial tension low).

Analysis of the mechanism of the phenomena herein described will be given in the paper following.

Some of the oil samples used in these experiments were given us by Dr. W. T. Bovie of the Harvard Biophysics Laboratories, whom we take this occasion to thank.

EXPLANATION OF PLATES.

The fine definition and wealth of detail which can be brought to the observer's eye, for reasons indicated above are necessarily lost in photography of these preparations.

PLATE 23.

FIG. 1. Cyclohexane-distilled water-*Bacterium coli*. × 138. Preparation photographed within 3 hours. The adsorbed bacteria form a semisolid band in the interface, appearing in the figure as a bright, heavily stippled line. Note the straight interface. Bacteria form heavy suspension in water; are almost absent from organic phase.

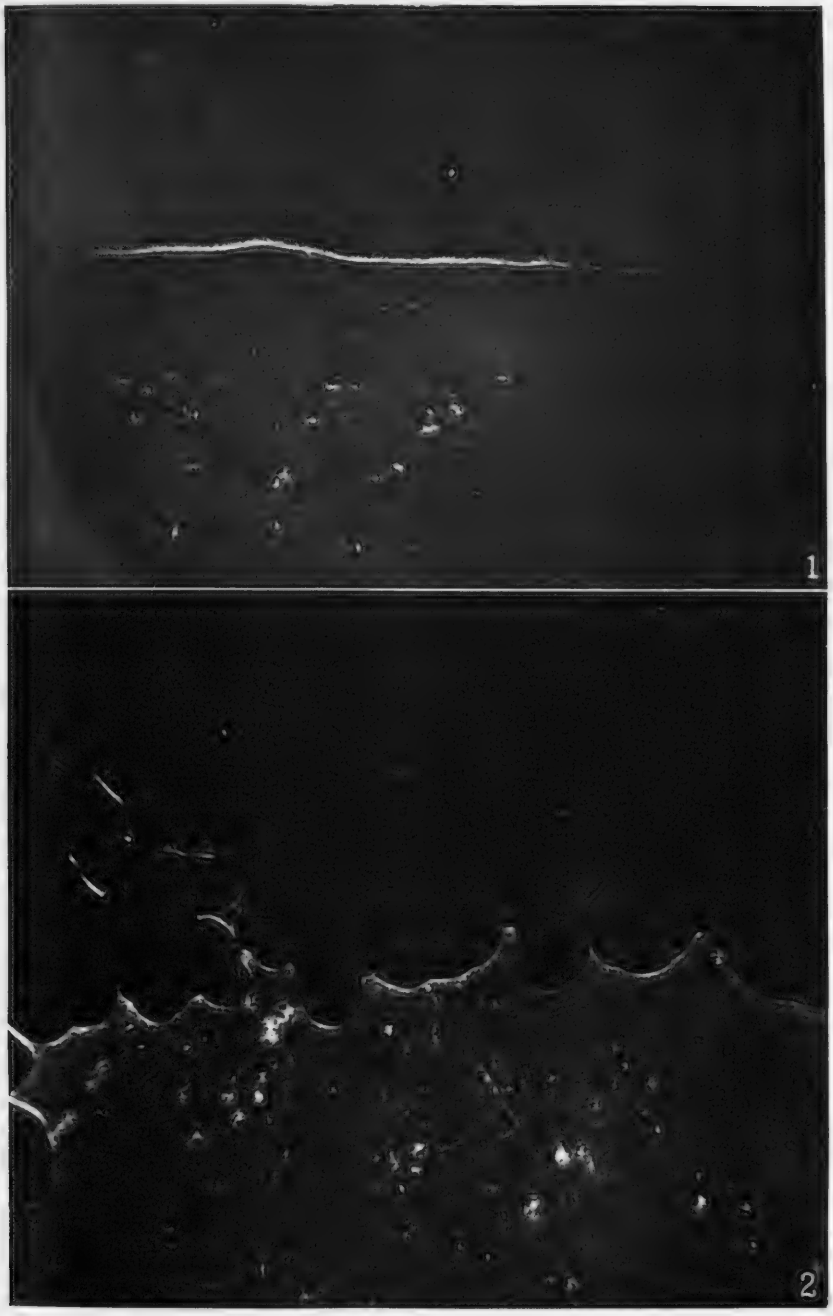
FIG. 2. Cyclohexanol-distilled water-*Bacterium coli*. × 138. Preparation photographed after about 3 hours. Sharply scalloped interfacial line. Less adsorption of bacteria in interface than with cyclohexane.

PLATE 24.

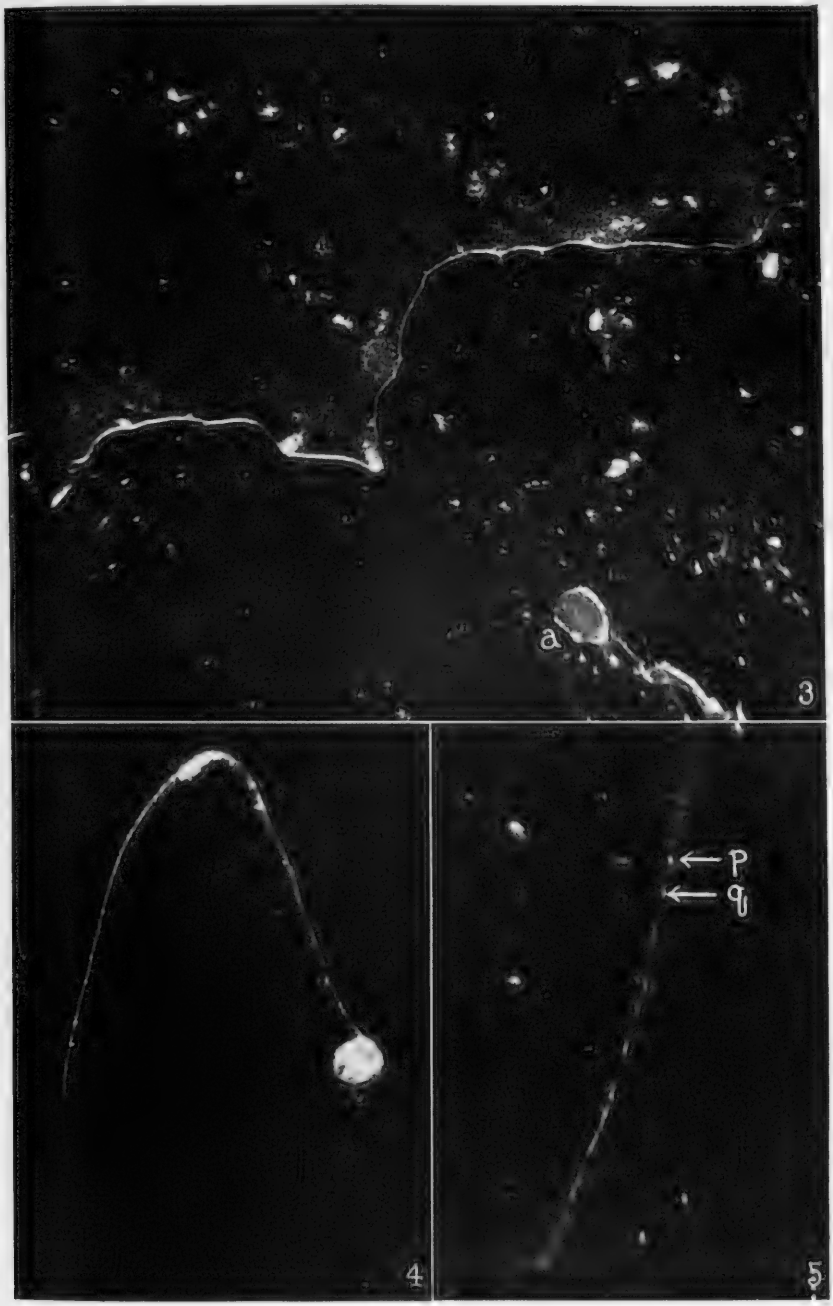
FIG. 3. Sperm oil-distilled water-*Bacterium coli*. × 138. Preparation photographed after about 24 hours. A vacuole of water containing microorganisms is shown enclosed in the oil at *a*. Bacterial individuals and clumps appear as bright specks.

FIG. 4. Cyclohexane-distilled water-*Bacterium coli*. × 292. Fresh preparation. Bacteria in interface give beaded appearance. Plate underexposed for bacteria in hydrocarbon and water phases.

FIG. 5. Cyclohexane-distilled water-*Bacterium coli*. × 625. Fresh preparation. Enlargement from moving picture film. Bacteria moving along interface appear as streaks, as do several undergoing brownian movement in water. Bright specks at *p* and *q* are bacteria in interface stuck to glass.



(Mudd and Mudd: Penetration of bacteria IV.)



(Mudd and Mudd: Penetration of bacteria. IV.)

CERTAIN INTERFACIAL TENSION RELATIONS AND THE BEHAVIOR OF BACTERIA IN FILMS.

By STUART MUDD, M.D., and EMILY B. H. MUDD.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Phenomena exhibited by motile and by acid-fast microorganisms in two-phase liquid films are described in this communication. Considerations of interfacial surface tension which have proven successful in the hands of other workers with somewhat allied systems are applied to bacteria in simple and modified films. It is shown that theory and observation are in agreement, although in the absence of means of evaluating solid-liquid interfacial tensions a rigorously quantitative test is not possible. The technique employed is found to give an experimental means of discriminating between existing theories concerning the surface tension factor in phagocytosis.

Theoretical.

The principles involved are set forth in a comprehensive article on capillary phenomena by J. Clark Maxwell.[1]

"If three fluids which do not mix are in contact with each other, the three surfaces of separation meet in a line, straight or curved. Let *O* [Text-fig. 1] be a point in this line, and let the plane of the paper be supposed to be normal to the line at the point *O*. The three angles between the tangent planes to the three surfaces of separation at the point *O* are completely determined by the tensions of the three surfaces.

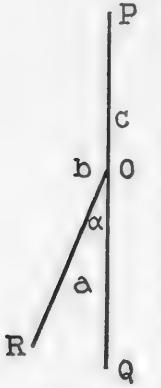
. . . . . .

"When a solid body is in contact with two fluids, the surface of the solid cannot alter its form, but the angle at which the surface of contact of the two fluids meets the surface of the solid depends on the values of the three surface-tensions. If *a* and *b* are the two fluids and *c* the solid, then the equilibrium of the tensions at the point *O* depends only on that of thin components parallel

[1] Maxwell, J. C., Capillary action, in Encyclopædia Britannica, Cambridge, 11th edition, 1910–11, v, 262.

to the surface, because the surface-tensions normal to the surface are balanced by the resistance of the solid. Hence if the angle *ROQ* at which the surface of contact *OR* meets the solid is denoted by α,

$$T_{bc} - T_{ca} - T_{ab}\cos\alpha = 0$$



TEXT-FIG. 1.

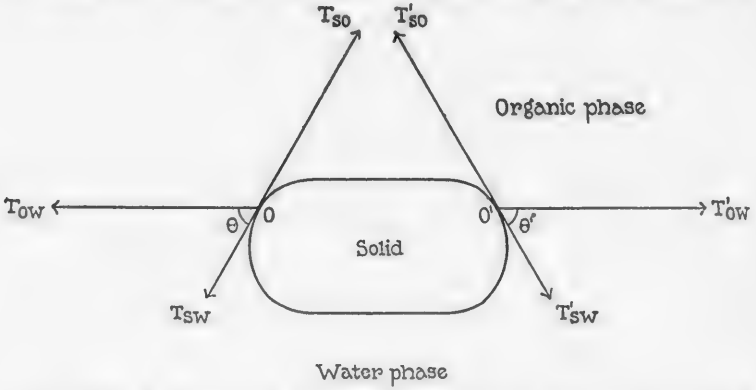
Whence

$$\cos\alpha = (T_{bc} - T_{ca})/T_{ab}$$

. . . . . .

"If the tension of the surface between the solid and one of the fluids exceeds the sum of the other two tensions, the point of contact will not be in equilibrium, but will be dragged towards the side on which the tension is greatest. If the quantity of the first fluid is small it will stand in a drop on the surface of the solid without wetting it. If the quantity of the second fluid is small it will spread itself over the surface and wet the solid. The angle of contact of the first fluid is 180° and that of the second is zero.

"If a drop of alcohol be made to touch one side of a drop of oil on a glass plate, the alcohol will appear to chase the oil over the plate, and if a drop of water and a drop of bisulphide of



TEXT-FIG. 2. Diagram of interfacial tensions acting at points *O* and *O'* in periphery of solid particle at equilibrium in interface between two immiscible liquids. The arrows by their direction and length indicate the direction and intensity of interfacial tensions at points *O* and *O'*. The solid-liquid tension at any other point could be indicated by arrows of proper length tangent to the solid at that point. θ is the contact angle made by the water phase with the solid.

carbon be placed in contact in a horizontal capillary tube, the bisulphide of carbon will chase the water along the tube. In both cases the liquids move in the direction in which the surface-pressure at the solid is least."

Let us consider now a bacterium in the interface between organic and aqueous phases (Text-fig. 2).

Let T_{so} be the tension in the solid (bacterial)—organic phase interface, T_{sw} that in the solid-water interface, and T_{ow} that in the interface between aqueous and organic phases.

At equilibrium,

$$T_{so} = T_{sw} + T_{ow} \cdot \cos\theta,$$

and similarly

$$T'_{so} = T'_{sw} + T'_{ow} \cdot \cos\theta' \tag{1}$$

Suppose now other values of the several tensions, so that[2]

$$T_{so} > T_{sw} + T_{ow} \tag{2}$$

Evidently the line of contact O will be dragged toward the organic side until the particle is completely wetted by and enveloped in the water phase. If

$$T_{sw} > T_{so} + T_{ow} \tag{3}$$

the particle will be wetted and enveloped by the organic phase.

Thus the necessary conditions of stable equilibrium for the particle in the interface are that

$$T_{so} < T_{sw} + T_{ow}$$

and

$$T_{sw} < T_{so} + T_{ow} \tag{4}$$

It follows then that

$$T_{sw} + T_{ow} - T_{so}$$

and

$$T_{so} + T_{ow} - T_{sw}$$

are in a sense measures of the stability of the particle in the interface. Explicitly, $T_{sw} + T_{ow} - T_{so}$ is the intensity of the force required to envelop the particle completely in the water phase and $T_{so} + T_{ow} - T_{sw}$ is the intensity of the force required to envelop the bacterium in the organic phase.

---

[2] It is assumed in this discussion for simplicity that $T_{sw} = T'_{sw}$ and $T_{so} = T'_{so}$.

Only the liquid-liquid interfacial tensions, T_{ow}, can at present be given numerical values. Unfortunately means are not available for evaluation of the solid-liquid tensions. Certain relations of interest can be inferred indirectly, however, and they will be found in harmony with the observational data.

Behavior of Motile Bacteria in Films.

The method of studying the behavior of microorganisms in two-phase films described in the foregoing paper[3] may be altered by using as aqueous phase broth or hay infusion cultures of motile bacteria. The technique is otherwise the same. Under these circumstances the bacteria may be observed while subject simultaneously to the restraining and kinetic forces of the interfacial surface tensions, and the driving force of their own flagella; the former are, of course, however, somewhat modified by the presence of surface-active substances in the media.[4] The forces thus acting on the bacteria prove to be of the same order of magnitude, and capillarity or motility may prevail, successively, according to circumstances.

The protocol of an individual experiment may be given.

Experiment 1.—Film of cedar oil against a 20 hour broth culture of a virulent mouse typhoid bacillus.[5] The interface was seen as a bright line with the usual interference fringes; bacteria were swimming actively about in aqueous phase. When first viewed, a strong movement of inert particles along the interface was occurring. As bacteria swam into the interface their anterior ends projected a little into the oil, but they were promptly turned about so that their long axes lay parallel to the line of the interface. They then swam very swiftly "down stream" or "up stream" slowly against the interfacial "current," making progress, being carried backward, or remaining stationary, according to the balance between their own motility and the interfacial streaming. At one time, six bacteria were observed in the interface like so many salmon in a riffle, undergoing the vibratory movements characteristic of bacteria in rapid locomotion, but making no progress against the current. Dead bacteria and other inert particles in the interface passed them rapidly. Several bacteria were observed swimming out of the interface into the water phase. The rule was to stay in the interface when once there, however. None were observed leaving the interface to pass into the oil.

[3] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633.

[4] Larson, W. P., Cantwell, W. F., and Hartzell, T. B., *J. Infect. Dis.*, 1919, xxv, 41.

[5] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

The angle of approach of bacteria entering the interface and the relative velocities of bacterial and interfacial movement determined whether the anterior end was directed up or down stream. Orientation down stream was naturally of more frequent occurrence. Bacteria swimming up to and apparently against the interface in this experiment and the others of the series were not always trapped there—often they swam immediately away.

Similar, though less striking results were obtained with *Vibrio percolans* and *Bacillus subtilis*. The following oils were used: cedar, peanut, sheep's foot, sperm, and mineral (liquid petrolatum Squibb) and melted vaseline (petroleum jelly, Chesebrough Co.). The vaseline was at first spread as a semisolid film against which the bacteria swam much as if against the glass. When the vaseline melted in the heat of the dark-ground illuminator, a liquid-liquid interface was formed in which the bacteria were trapped, along which they moved, and from which they escaped into the water much as with the oil-water interfaces of high tension.

Cyclohexane, cyclohexanol, oil of sassafras, oil of lavender, and 1 per cent phenol in albolene immobilized the bacteria near the interface, an ordinary non-motile experiment resulting.

A peculiarly vivid picture of the action of the interfacial trapping and kinetic mechanisms was obtained in these experiments. It is also noteworthy that when the bacteria threshed their way out of the interface, escape was always into the aqueous phase, and never, in the fourteen experiments performed, was escape into the oil noted. This would correspond to the condition that

$$T_{so} + T_{ow} - T_{sw} > T_{sw} + T_{ow} - T_{so},$$

or

$$T_{so} > T_{sw}$$

Behavior of Acid-Fast Microorganisms in Films.

Evidence has been adduced by Deycke and Much and by Leschke[6] to indicate that the acid-fast microorganisms are coated with a mixture of fatty acids and a neutral fat or fats. There is good reason to believe, on the other hand, that the membrane surface of ordinary

[6] Leschke, E., *Beitr. Klin. Tuberk.*, 1911, xx, 400.

bacteria contains very many active or polar groups ($-COOH$, $-NH_2$, $-OH$, etc.).[7] Hence the ordinary bacteria should be more readily wet by water, the polar liquid, and the acid-fast microorganisms by the non-polar organic liquids. It therefore seemed likely that the behavior of the two groups of microorganisms in films might differ detectably. This has proved indeed to be the case. The acid-fast bacteria pass through the interface and into the organic phase with a readiness in marked contrast to the behavior of ordinary bacteria already described.

Acid-fast bacteria are present in much smaller numbers in the interfaces, for the reason that they are in an equilibrium of low stability there or not in equilibrium at all. Sliding movements up and down the interface are correspondingly reduced, and in the case of oleic acid-distilled water and some cedar oil-distilled water interfaces it may be almost entirely suppressed; interfacial forces may carry the acid-fast organisms into the oleic acid phase directly they enter the interface. A more typical behavior is for the individual bacterium in the water phase, when overtaken by the advancing interface, to slide along the interface for a greater or less distance before being completely imported into the organic phase with the aid of some mechanical work. This mechanical work is similar in kind, although very much less in degree, to that described in the foregoing paper in connection with ordinary bacteria.

When an organic phase is slowly encroaching on a water phase and the interface reaches a mat of acid-fast bacteria stuck to the glass, the interface usually runs ahead locally of the general line of advance and wets the group of acid-fast bacteria, enclosing many of them forthwith in the organic phase. The long bays and peninsulas drawn out behind an interface advancing on an aqueous suspension of ordinary bacteria, which the organic phase does not readily wet[8] (see Figs. 1 and 3), are correspondingly infrequent with the acid-fast preparations. Such peninsulas may be formed, however, when the interface is pulled out by some of the granular detritus found especially in old cultures of the acid-fast organisms.

[7] Rideal, E. K., Colloid phenomena in bacteriology, *Brit. Assn. Advancement Sc., 5th Rep. on Colloid Chemistry*, 1923, 31.

[8] Mudd and Mudd,[3] Figs. 1 and 3.

Brownian movement in the interfaces has been noted as in general freer with the acid-fast than with the ordinary bacteria.

Acid-fast bacteria may be suspended in a number of the organic phases, *e.g.* mineral oil, cedar oil, olive oil, oleic acid, cyclohexanol. When such preparations were studied in films against distilled water, acid-fast bacteria were usually soon seen suspended in the aqueous phase also. Apparently they reached the aqueous phase with the aid of mechanical work in the same manner described for passage of ordinary bacteria in the reverse direction.[3]

In three of four experiments with mineral oil, no bacteria were observed to pass into the water, although much of the granular débris soon reached the aqueous phase.

Four strains of non-pathogenic Saranac Lake strains of acid-fast bacteria were used in these experiments, the smegma bacillus (*Mycobacterium smegmatis*), butter bacillus (*Mycobacterium berolinensis*), mist bacillus (*Mycobacterium stercusis*), and milk bacillus. They were grown on 5 per cent glycerol agar.

Olive oil, cedar oil, oils of lavender and sassafras, liquid albolene, mineral oil (liquid petrolatum Squibb), vaseline (petroleum jelly, Chesebrough Co.), isoamylic and isobutylic alcohols, cyclohexanol, cyclohexane, and oleic acid were used as organic phases.

Illustrative protocols may now be given.

Experiments 2 and 3.—Smegma bacillus and butter bacillus. Cultures had been kept sealed 3 months on 5 per cent glycerol agar. Films of oleic acid against distilled water suspension of acid-fast bacteria.

Throughout the observations, the organic liquid advanced slowly or rapidly on the aqueous bacterial suspension. Many bacteria in the water phase were adherent to the glass. As the interface reached and began to pass over such an adherent bacterium it was frequently torn loose from the glass and shot through into the organic phase. Floating bacteria were similarly swiftly imported into the organic phase. Floating granules were usually trapped in and underwent streaming movements along the interface. As the slowly moving interface advanced against a bacterium stuck to the glass in the aqueous phase, the interfacial line could frequently be seen to advance on the bacterium as a liquid does upon a solid it wets, and to accelerate the local advance of the organic phase. An occasional bacterium was observed to slide down the interface. Such were probably stuck to particles which were trapped in the interface.

Obviously, $T_{sw} > T_{so} + T_{ow}$. This expression represents the condition under which the organic phase tends to spread on the bacteria and displace the water.

Experiment 4.—Film of petroleum jelly against distilled water suspension of milk bacillus. When first observed, bacteria were to be seen in brownian movement in water, motionless in interface, and stuck to glass in vaseline phase. After a time the interface began to advance slowly toward the organic phase side. The bacteria in the boundary line moved slowly along the interface, for a short distance or through several low power fields, before passing by almost imperceptible degrees, floating through as it appeared, into vaseline phase. There was no brownian movement of bacteria in the interface. One droplet of vaseline was observed to approach a mat of bacteria adherent to the glass. The interface then ran out on the mat wetting it and engulfing some of the bacteria. The boundary line was observed to overtake floating mats of bacteria. These it scattered along the interface, some traveling in each direction from the site at which contact with the interface was first made. Stability in the interface seemed to exist, but the bacteria moved very readily into the organic phase.

The following conditions seem to obtain.

$T_{sw} < T_{so} + T_{ow}$ slightly
$T_{so} < T_{sw} + T_{ow}$
$T_{sw} > T_{so}$

} The organic phase tends to spread on the bacteria and is prevented from so doing, if at all, only by the liquid-liquid interfacial tension.

Experiment 5.—Film of distilled water against suspension of butter bacteria in cyclohexanol. Bacteria were suspended in alcohol and water was allowed to run in from the other side of the cover-slip. Bacteria were swept in masses on the organic side of the retreating interface and held as against a wall except when an extremely active sweeping movement of phases or sliding up and down the interface shot bacteria over into water. When masses that stuck to the glass under alcohol were overtaken by the interface, they often became suspended in the water, gradually being dispersed to form an even suspension. Bacteria were seen trapped in and sliding up and down the interface but these were in relatively small numbers. When phases were nearly motionless, no bacteria were seen to pass from alcohol into water or *vice versa*. The general impression was of a strong trapping mechanism which prevented bacteria from passing from alcohol into water without violent work on them, or from water into alcohol without some work.

Bacteria in Modified Films.

It is clear that it should be possible, by adding substances which will decrease the liquid-liquid interfacial tension, to shift the picture presented by the group of liquids with high interfacial tension against water toward that of the low interfacial tension group. This again proves to be the case. As has already been indicated, preparations of mineral oil or cyclohexane against distilled water

suspensions of ordinary bacteria show relatively straight interfaces, containing a high concentration of bacteria which reach the oil phase only with the aid of mechanical work of extreme degree. A suspension of bacteria in 0.06 per cent sodium oleate solution against mineral oil, on the other hand, shows interfaces more readily drawn out into peninsulas, containing fewer bacteria, and the microorganisms escape into the oil with the aid of less extreme mechanical work.

This effect of soap is, of course, not specific. A modification of the typical picture may similarly be occasioned by inadequately cleaned slides or cover-slips, and sufficient surface-active material from the bacterial culture may be added in making the suspension to cause a noticeable alteration from typical behavior. Differences in different cultures in the amount of surface-active constituents they contain seem to exist to detectable degree. It is to be noted, however, that neither by accident nor by design have we found preparations in which non-acid-fast bacteria pass by surface tension alone out of the interface. Some bacteria are always trapped in the interface, in equilibrium as regards the two phases, apparently, until acted upon by sufficient mechanical force to displace them into one or the other phase. And some sliding up and down the interface is almost invariably to be detected.

Modification of the preparation as a result of accidental contamination in such a way as to increase bacterial stability in the interface has also been noted. In one series of six experiments with distilled water suspensions of acid-fast bacteria against mineral, olive, and lavender oils, isobutylic alcohol and oleic acid (two experiments), the bacterial stability in the interface and the difficulty of ingress into the organic phase resembled preparations of ordinary bacteria and were unlike forty odd other experiments with acid-fast microorganisms. The difficulty was here traced to contamination of the slides used by a little dirty water in the bottom of the containing vessel.

Interpretation.

The possibility of explaining the observations on bacteria in interfaces by the theoretical interfacial tension relations may now be examined.

Suppose, first, that unequal diffusion of surface-active substances, unequal heating of the film, or other factor, decreases the water-organic phase tension on one side of the bacterium below that on the other, *i.e.* T_{ow} becomes greater or less than T'_{ow}. The bacterium will evidently tend to be dragged along the interface in the direction of the stronger interfacial tension. Thus if the free surface energy of the interface is not uniformly distributed, the particles trapped, "absorbed," in the interface will tend to accumulate in higher concentration in regions of greater interfacial tension than in regions of less tension. This mechanism was probably the chief means of effecting the streaming movements or "spreading" along the interface so evident in practically all systems studied in this and the foregoing paper, although we believe that movements due to evaporation from the margin of the film, mixing of the phases, etc. also played a part. Undoubtedly encroachment of one phase on another because of the first having greater wetting power for the glass, as described by Maxwell for carbon bisulfide and water, is partially responsible for the movements of the phases as a whole.

The interfacial kinetic mechanism just described was exhibited in greater or less degree in all the systems examined. The individual characteristics of the preparations will now be considered under several type cases.

Case A.—Ordinary non-motile or motile bacteria. The liquid-liquid tension, T_{ow}, is high.

Examples: *B. coli* or *B. subtilis* in interface between distilled water and cyclohexane or mineral oil. Motile *Vibrio percolans* or *B. pestis caviæ* in the interface between medium and mineral oil.

Since T_{ow} is large, the conditions of stability must obtain unless there is great disparity between T_{sw} and T_{so}. Such a disparity evidently does not exist in systems of this class, since the bacteria concentrate in the interface and only leave it when considerable mechanical work is done upon them. For this case, therefore,

$$T_{so} < T_{sw} + T_{ow},$$

and

$$T_{sw} < T_{so} + T_{ow}$$

Case B.—Ordinary bacteria. The liquid-liquid tension, T_{ow}, is low.

Examples: *B. coli* or *Vibrio percolans* in the interface between distilled water with cyclohexanol, isoamylic or isobutylic alcohols.

Stability should be much less than in Case A. As the limiting case is approached were $T_{ow} = O$, and the liquids become completely miscible, the bacterium can only be in surface tension equilibrium in the interface, provided T_{sw} practically equals T_{so}. In agreement with theory, the bacteria are found in much lower concentration in the interface than in Case A and they escape more readily into either water or organic phase than when T_{ow} is high.

The facts regarding behavior of bacteria in the systems of Case A and Case B were observed and recorded before we had an adequate explanation of them. The later cases were in part predicted from theory.

Case C.—Acid-fast bacteria.

Examples: Smegma, milk, butter, or mist bacteria in films of oleic acid, mineral oil, cyclohexane, cyclohexanol, etc. against water.

Bacterial surface layer of fatty material. Since mutual solubility or miscibility and low interfacial tension go hand in hand, and immiscibility is correlated with high interfacial tension,[9,10] T_{so} should be relatively small and T_{sw} large. Thus $T_{so} + T_{ow} - T_{sw}$ should be reduced to a low or even negative value.

Again, in agreement with expectation, the bacteria have extremely low stability in the interfaces, pass readily into the organic phases, and in oleic acid-water preparations at least are often thrust into the organic phase as soon as they come into contact with the interface.

Case D.—Ordinary bacteria. Liquid-liquid tension, T_{ow}, high in control, reduced by surface-active substance in experiment.

Examples: *B. subtilis* in interface between mineral oil and 0.06 per cent Na oleate solution. *Vibrio percolans* in interface between cyclohexane and 0.06 per cent Na oleate solution.

Theory requires shift of picture from that characteristic of Case A in the direction of that of Case B.

Experiment verifies expectation.

DISCUSSION.

Among the most important and fruitful attempts to explain phagocytosis in physical terms is that of Rhumbler, who attributed it almost wholly to the effect of interfacial molecular attractions at the cell surface. Although subsequent work has served to emphasize the importance of phenomena within the phagocytic cell, such, for

[9] Harkins, W. D., Brown, F. E., and Davies, E. C. H., *J. Am. Chem. Soc.*, 1917, xxxix, 354.

[10] Coghill, W. H., and Anderson, C. O., Certain interfacial tension equilibria important in flotation, *U. S. Bureau of Mines, Techn. Paper 262*, 1923.

instance, as changes in the consistency of its protoplasm,[11–14] nevertheless the interfacial tension relations are of capital importance and deserve accurate statement. Rhumbler's formulation of the surface tension factor in phagocytosis, therefore, requires consideration. His *Importgesetz* and *Exportgesetz* state essentially that if a particle reaches the interface between two fluids it will be taken into that fluid for which it possesses the greater adhesion, provided that this adhesion is greater than the cohesion of the fluid in question.[15]

Consideration of Text-fig. 2 will show that this formulation is inadequate. It follows from the second law of thermodynamics that at equilibrium the particle will be so disposed as to give the system a minimal free energy, whether this equilibrium position is in one or the other liquid phase or in the interface between the phases. If the particle leaves the interface, the area of the liquid interface will be increased by some amount, y, and an increment of free energy, $y \cdot T_{ow}$, thereby made. Any formulation such as Rhumbler's which wholly leaves out of account T_{ow} is therefore inadequate on theoretical grounds. It is, in part, contradicted by our experiments, furthermore, which indicate a high degree of stability of the bacteria in the interface in many systems studied and correlation of that stability with the liquid-liquid tension, T_{ow}.

Tait[16] improved somewhat upon Rhumbler's formulation, but similarly supposed that the foreign particle must necessarily be carried by surface tension into one or the other phase.

Fenn[12] has criticized Tait's equations, and has himself formulated admirably the interfacial forces involved in the adhesion of a hypothetical fluid cell to a solid surface and in the ingestion of a solid particle by the cell. In this paper we are concerned only with the latter case. Fenn shows both from considerations of free interfacial energy and of interfacial tension that the cell will only completely ingest the

[11] Loeb, L., *Am. J. Physiol.*, 1921, lvi, 160.

[12] Fenn, W. O., *J. Gen. Physiol.*, 1921–22, iv, 373.

[13] Barr, C. E., *J. Med. Research*, 1923, xliv, 79.

[14] Chambers, R., The physical structure of protoplasm as determined by micro-dissection and injection, General cytology, Chicago, 1924, Section V.

[15] Rhumbler, L., *Ergebn. Physiol.*, 1914, xiv, 577, 581.

[16] Tait, J., *Quart. J. Exp. Physiol.*, 1918–20, xii, 1.

particle unless other than capillary forces intervene, when the conditions of complete spreading of cell on particle are fulfilled. That is, if we call the cell the organic phase, the plasma the aqueous phase, and the particle *s*, ingestion is only complete if

$$T_{sw} > T_{so} + T_{ow}$$

If conditions of partial spreading obtain, the particle will be only partially ingested, *i.e.* will be in equilibrium in the plasma-cell interface.

It is clear then that our observations and formulation contradict those of Rhumbler and of Tait and substantiate the statement of Fenn.

For valuable data on and discussion of surface tension effects in somewhat allied systems, reference is made to papers by Harkins and Feldman,[17] Coghill and Anderson,[10] and the monograph of Willows and Hatschek.[18]

SUMMARY.

To account for the behavior of a solid particle in the interface between two fluids it is necessary to consider, as indicated by Clark Maxwell, three surface tensions: T_{so}, the tension in the interface between the solid particle and the organic phase; T_{sw}, the tension in the interface between solid particle and aqueous phase; and T_{ow}, the organic phase-water interfacial tension.

If $T_{so} > T_{sw} + T_{ow}$, (2), the stronger solid-organic phase tension should pull the line of intersection of the three phases around the periphery of the solid particle until the particle is completely enveloped in the *water* phase.

If $T_{sw} > T_{so} + T_{ow}$ (3), the solid-water tension should pull the line of meeting of the phases about the particle until it is enveloped in the *organic* phase.

If

$$T_{so} < T_{sw} + T_{ow}$$
$$T_{sw} < T_{so} + T_{ow} \qquad (4)$$

[17] Harkins, W. D., and Feldman, A., *J. Am. Chem. Soc.*, 1922, xliv, 2665.

[18] Willows, R. S., and Hatschek, E., Surface tension and surface energy and their influence on chemical phenomena, Philadelphia, 3rd edition, revised and enlarged, 1923.

the particle should be stable in the interface, only leaving it when mechanical work overcomes the equilibrium due to the balance of interfacial tensions.

The ordinary bacteria used have been stable in the interfaces between water or aqueous solutions and all organic liquids tested; *i.e.*, condition (4) obtains. In preparations in which T_{ow} is large, stability has been found by experiment to be greater than when T_{ow} is small, as follows from condition (4).

The force, dependent upon condition (4), which holds bacteria in the liquid-liquid interface, and the force, dependent upon unequal distribution of tension in the liquid-liquid interface, which causes bacteria to glide along the interface, prove to be of the same order of magnitude as the force due to bacterial flagella. Interfacial tensions or its own motility may dominate the movement of the bacterium, according to circumstances. When bacteria thresh their way out of the interface, escape is into the aqueous phase.

Acid-fast bacteria possess very low or, in some cases, no stability in the interface, passing easily or even spontaneously into the organic phase.

Good evidence has been advanced by other workers to indicate that the surfaces of ordinary bacteria contain many polar radicals; on the other hand, the acid-fast microorganisms are coated with predominantly non-polar substances. It follows from known principles, therefore, that T_{so} should be greater than T_{sw} with ordinary bacteria, and T_{sw} should be greater than T_{so} with acid-fast bacteria. Consideration of relations (2) and (3) above will show that these conditions should result in the differences in behavior of acid-fast and ordinary bacteria actually found by experiment.

The theoretical and experimental data here developed contradict the theoretical formulations of the surface tension factor in phagocytosis advanced by Rhumbler and by Tait and substantiate those of Fenn.

TISSUE RESISTANCE AND THE CAUSE OF PERMANENT ACQUIRED IMMUNITY.*

By S. NUKADA and T. MATSUZAKI.

(*From the Laboratories of Tokyo Imperial University, Tokyo, Japan, and Peking Union Medical College, Peking, China.*)

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It is well known that a single attack of certain infectious diseases protects an individual against subsequent infection, that is to say, there develops a permanent acquired immunity. To this group of diseases belong smallpox, scarlet fever, measles, typhoid fever, cholera, etc., while in such infectious diseases as gonorrhea, diphtheria, recurrent fever, influenza, pneumonia, etc., the patient acquires no lasting immunity. Immunity can be produced not only by natural infection, but also by artificial means. There are two kinds of immunity, one against infection, that is, antibacterial, and the other against toxin, that is, antitoxic.

According to the modern theory, acquired immunity, whether antibacterial or antitoxic, permanent or not, is due principally to the existence in the blood of specific antibodies, such as bacteriolysins, opsonins, bacteriotropins, antiaggressins, agglutinins, precipitins, and antitoxins. It is generally supposed that of these various antibodies bacteriolysins, opsonins, bacteriotropins, and antiaggressins are the most important in producing immunity against infection, the presence of agglutinins and precipitins being rather a phenomenon following upon the immunization, while antitoxins are considered as mere protective substances against toxins.

Whether or not the presence of antibodies in the blood has anything to do with immunity, it is at least generally admitted that there is no relationship between the degree of immunity and the quantity of

* A preliminary report was read before the meeting of the Tokyo Medical Society, February 20, 1923.

specific antibodies in the blood. For instance, in typhoid fever, immunity persists throughout life, even after antibodies have disappeared from the blood.

The permanent acquired immunity which remains after antibodies have disappeared from the blood is usually explained by the supposition that at the second infection specific antibodies are more quickly produced than normally. Liebermann and Acél[1] observe, however, that following a second infection of typhoid-immune animals, from the blood of which specific antibodies have disappeared, antibodies appear in the blood only after several days, although the animals withstand such a virulent inoculation as would kill normal animals within 24 hours, a fact which cannot be accounted for by the theory of antibodies. The presence of antibodies in the blood, therefore, though an interesting serological phenomenon, does not in itself mean immunity, at least in the case of permanent acquired immunity against infection. This fact has led us to turn our attention to the tissues of typhoid-immune animals and to conduct researches with them. Our researches differ in intent from those upon acquired immunity against toxin, as recently reported by Gunn and Heathcote[2] and by Manwaring and Williams[3] with cobra venom.

We have made the following experiments in order to determine (1) whether the tissues of typhoid-immune animals acquire increased resistance against the toxic products of typhoid bacilli, (2) whether this resistance, if acquired, is specific or not, and (3) whether the increased resistance is caused by antitoxin or by a specific biological alteration of the tissue cells.

Methods.

The experiments were made on the hearts of rabbits. We found the heart most suitable for the purpose, because after isolation from the body it can with comparative ease be freed entirely of blood, and it beats automatically for a long time under suitable conditions.

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[1] von Liebermann, L., and Acél, D., *Deutsch. med. Woch.*, 1917, xliii, 867.

[2] Gunn, J. A., and Heathcote, R. S. A., *Proc. Roy. Soc. London, Series B*, 1921, xcii, 81.

[3] Manwaring, W. H., and Williams, T. B., *J. Immunol.*, 1923, viii, 75.

(*a*) *Preparation of Animals.*—The rabbits were adult, of about 2,000 gm. weight, and mostly male. They were kept in cages under controlled conditions, and were all fed on the same diet. Care was taken to see that the quality and quantity of food were sufficient in every case to prevent diminution of the weight of animals.

The animals were immunized actively with vaccine which was in almost all cases injected into the marginal veins of the ears and in only a few cases subcutaneously giving a total of five injections at intervals of 5 to 7 days. The doses were progressively increased from $\frac{1}{4}$ to $\frac{1}{2}$, 1.0, 1.0, 1.0 mg. per kilo. All vaccines contained 1.0 mg. of bacteria per 1.0 cc. They were freshly prepared in the usual manner, and were sterilized by heating for 1 hour at 60°C.

Using the Langendorff-Locke apparatus, the isolated heart was perfused with Locke-Ringer solution for at least 10 minutes to remove all serum from the heart, and then the toxic solution was tested. The heart beats were registered on the smoked paper of a kymograph.

(*b*) *Preparation of a Solution Containing Toxic Products of Typhoid Bacilli.*—It is absolutely necessary that the toxic products do not lose the specificity of typhoid bacilli, yet they must be notably toxic at the same time. It is quite difficult to obtain a toxic solution, as the toxic substance in typhoid bacilli belongs to the so called endotoxin group. We tried several methods of preparation and finally found the following one most suitable for the experiments. Highly virulent typhoid bacilli were cultured in 2 per cent peptone water (20.0 gm. peptone + 9.2 gm. NaCl + 1,000.0 cc. aqua destillata) for 7 to 10 days in an incubator. The material was then heated for 1 hour at 60°C., again kept in an incubator for about 10 days, to permit the endotoxin to pass by digestion into the peptone water from the bacilli, and was finally stored in a dark cool place.

Prior to the experiments, after the toxic solution had been cleansed of sediment by filtration, salts were dissolved in it in the same proportion as in Locke-Ringer solution. Finally this toxic solution was diluted 200 to 400 per cent with Locke-Ringer solution freshly prepared.

The toxicity of the solution obtained in this manner is very variable. Therefore we have tested each lot of material upon the isolated heart of a normal rabbit. According to our experience, a toxic solution which stops the normal heart in several minutes, is most suitable for the experiment. If it is either too strong or too weak it is not suitable.

The total quantity required to stop the heart beat is naturally variable. In Experiment 6 (Table I) about 500 cc. were used in the course of 25 minutes, yet the heart still beat.

Each set of experiments has been carried out with the same toxic solution, and with an identical procedure and technique for the hearts.

EXPERIMENTS AND RESULTS.

(*a*) *Comparison of the Resistance.*—We compared first the resistance of the hearts of typhoid-immune rabbits with that of normal non-immunized animals against toxic products of typhoid bacilli.

Table I shows clearly that the resistance of the hearts of typhoid-immune rabbits, examined between the 9th and 58th days after the

TABLE I.

No. of experiment.	Condition of rabbit.	No. of days between the last injection of vaccine and the experiment.	No. of min. during which the heart continued to beat.
1	Immune.	9	50
	Normal.		20
2	Immune.	9	45 (still beating).
	Normal.		4
3	Immune.	11	20
	Normal.		4
4	Immune.	11	15
	Normal (*a*).		4
	" (*b*).		14
5	Immune.	14	35
	Normal.		8
6	Immune (*a*).	14	25 (still beating).
	" (*b*).	58	25 (" ").
	Normal (*a*).		12
	" (*b*).		12
	" (*c*).		10

last injection of vaccine, is greater than that of the normal non-immunized rabbits as tested with the same solution of toxic products of typhoid bacilli.

(*b*) *Problem of Specificity.*—We next tried to compare the resistance to toxic products of typhoid bacilli of the hearts of typhoid-immune rabbits and those of animals immunized with bacteria other than typhoid bacilli.

TABLE II.

No. of experiment.	Bacteria used for immunization.	No. of days between the last injection of vaccine and the experiment.	No. of min. during which the heart continued to beat.
7	*B. typhosus.*	11	25
	" *paratyphosus* B.	11	13
	" " A.	11	10
	Non-immunized.		20
8	*B. typhosus.*	10	4.5
	" *coli.*	10	3.5
	Non-immunized.		3.5
9	*B. typhosus.*	11	20 (still beating).
	" "	11	15 (" ").
	" *dysenteriæ.*	11	10
	Non-immunized.		10
10	*B. typhosus.*	11	18
	" *pneumoniæ.*	11	40 sec.
	Non-immunized.		15
	B. typhosus.	12	15
	" *pneumoniæ.*	12	10 sec.
	Non-immunized.		7
11	*B. typhosus.*	11	18
	" *influenzæ.*	11	15 sec.
	Non-immunized.		15
	B. typhosus.	12	15
	" *influenzæ.*	12	10 sec.
	Non-immunized.		7
12	*B. typhosus.*	11	18
	" *pertussis.*	11	15 sec.
	Non-immunized.		15
13	*Diplococcus pneumoniæ.*	11	30
	Non-immunized.		30
14	*B. typhosus.*	11	7
	Diplococcus gonorrhœæ	11	6
	" "	11	2
15	*B. typhosus.*	12	23
	" *pestis.*	12	16
	Non-immunized.		10
16	*B. typhosus.*	10	20
	" "	10	20
	Vibrio choleræ.	10	20
	Non-immunized.		16

In Table II the resisting power of the hearts of typhoid-immune rabbits is shown to be greater than that of the hearts of rabbits immunized with bacteria other than typhoid bacilli. Therefore it is certain that the acquired increase of resisting power of the heart tissue of the typhoid-immune animals is specific.

(*c*) *The Cause of Increased Resistance.*—There are two possibilities: one, that the increased resistance is caused by antitoxin in the heart tissue, and the other that it is caused by a specific biological alteration of tissue cells. If the antitoxin cannot be demonstrated in the heart tissue, then the latter must be regarded as the cause.

The following experiments were performed to determine the presence or absence of antitoxin in the cardiac tissue of immunized animals.

TABLE III.

No. of experiment.	Condition of animal from the heart of which fluid was expressed.	No. of min. during which the perfused heart continued to beat.
17	Immune.	18
	Normal.	16
18	Immune.	12
	Normal.	12

In a first series of experiments the isolated hearts of normal rabbits were perfused with toxic solution which had previously been mixed with the fluids expressed from hearts, sometimes of immunized, sometimes of non-immunized animals. If antitoxin is contained in the heart tissue of typhoid-immune animals, the depressant action of a toxic solution mixed with the fluid from the heart tissue of typhoid-immune animals, ought to be less than that of the same toxic solution when mixed with the fluid from the hearts of normal animals.

There was, however, as Table III shows, no constant difference to be observed in the toxicity of solutions mixed with fluid from the hearts of immunized animals as compared with those mixed with fluid from the hearts of non-immunized animals. Thus we failed to detect antitoxin in the fluid from the heart tissue of typhoid-immune animals by means of this experiment.

We next conducted experiments to compare the antitoxic power of the fluid from the heart tissue of typhoid-immune animals with

that of normal heart tissue, by means of subcutaneous injection into mice of these fluids mixed with emulsion of typhoid bacilli. The fluid was injected in dosage of 1.0 cc. for every mouse of 10 gm. body weight. The fluid injected contained in every 1.0 cc. the fluid procured from 1.0 gm. of heart tissue.

As Table IV shows, the existence of antitoxin in the fluid from heart tissue of the immunized rabbits was also negatived by this experiment.

Taking into consideration (1) the results of these two kinds of experiments and (2) the fact that even in the blood the occurrence of antitoxin against endotoxin has never been definitely shown, we feel justified in concluding that the increased resistance is not due to antitoxin, but is caused by a specific biological alteration of the tissue cells.

TABLE IV.

No. of mouse.	Condition of animal from the heart of which fluid was expressed.	No. of hrs. of survival.
1	Immune.	20
2	"	45
3	"	45
4	Normal.	45
5	"	45
6	"	Living.

DISCUSSION AND CONCLUSION.

In these experiments, we have demonstrated that the tissue cells of the hearts of typhoid-immune animals are altered in such a way that their resisting power is increased specifically against toxic products of typhoid bacilli. From this it may reasonably be inferred that other tissue cells the resisting power of which is low in the non-immunized state, simultaneously acquire an increased resisting power. Once the tissue cells acquire increased resistance against toxic products of bacilli, the bacilli lose their power of injuring the tissue cells, and become like saprophytes which do no injury to the host. Even under this condition bacteria may of course invade, but they do not cause symptoms. Even if they enter the blood, some of them will soon be carried out of the body, while others will be caught

by phagocytes or destroyed by the bactericidal strength of the blood. It need scarcely be pointed out that since the normal blood has natural non-specific bactericidal strength, specific antibodies are not required in order to destroy the bacilli. Moreover, as stated at the outset, it is plainly impossible to ascribe the cause of permanent acquired immunity to the existence of antibodies in the blood.

On the strength of the results of our experiments, we cannot but maintain that permanent acquired immunity, at least in typhoid, instead of being principally caused by antibodies in the blood, is due rather to a lasting specific increase of resisting power acquired by the tissue cells against the toxic products of bacteria. This seems to hold true not only with typhoid, but also with other infectious diseases, in which one attack causes lasting immunity. Many facts related to permanent acquired immunity which have hitherto been found difficult to explain through the theory of antibodies are rendered intelligible by this theory of tissue resistance.

For example, specific antibodies in the blood, even bacteriolysins and opsonins, are produced not only by bacteria which leave behind permanent immunity, but also by those which do not. This fact cannot be explained away on the theory of circulating antibodies. It might be that while bacteria which do not cause permanent immunity by infection, alter tissue cells only temporarily or in a slight degree, those which convey permanent immunity after infection, alter the tissue cells permanently and to a much higher extent. Such a supposition would at least explain satisfactorily why permanent immunity cannot be acquired through infection by all varieties of bacteria.

Furthermore, the preventive effect of vaccine has been demonstrated, save of smallpox, only in those of certain infectious diseases in which permanent immunity follows an attack, such as typhoid, cholera and plague. In these cases specific antibodies appear after vaccine injection just as upon spontaneous infection, yet complete immunity is not acquired as a result, for the individuals or animals receiving vaccine injection are not absolutely protected against infection. This fact seems also to argue against the conception that antibodies are the cause of permanent acquired immunity against infection. If, on the other hand, the tissue resistance is taken into

consideration it becomes easy to explain the observed phenomena. One need only suppose that whereas by spontaneous infection the resisting power of tissue cells is increased permanently, by vaccine injection the tissue cells are altered only incompletely.

Finally it may be mentioned that the so called "local immunity" usually means a production of antibodies by the tissue cells with which the antigen comes into direct contact. This view is founded on the experiments by Wassermann and Citron[4] among others. The term "tissue immunity" is often understood as a synonym for local immunity. For such reason this term seems to be unsuitable as applied to the increase of resisting power acquired by tissue cells, because this is, as stated before, not caused by antitoxin and has no direct relation to the production of antibodies.

SUMMARY.

1. The resistance against toxic products of typhoid bacilli of the heart tissues of typhoid-immune animals is greater than that of non-immunized animals.

2. The acquired increase of resisting power is specific.

3. The increased resistance is not caused by antitoxin, but by a specific biological alteration of tissue cells.

4. It is maintained that permanent acquired immunity, at least in typhoid, instead of being due principally to antibodies, is caused by a lasting specific increase of resisting power acquired by the tissue cells against the toxic products of bacteria.

5. This theory seems to hold good with other infectious diseases in which one attack conveys permanent immunity.

[4] Wassermann, A., and Citron, J., *Z. Hyg.*, 1905, l, 331; *Deutsch. med. Woch.*, 1905, xxxi, 573.

ON THE NATURE OF THE HEAT-LABILE SUBSTANCE ("V") REQUIRED FOR THE GROWTH OF THE BACILLUS OF PFEIFFER.

By TH. THJÖTTA, M.D.

(*From the Bacteriological Laboratory of the Norwegian Army, Christiana, Norway.*)

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In previous communications it has been shown by Thjötta[1] and by Thjötta and Avery[2,3] that the bacillus of Pfeiffer needs for its growth two substances present in blood and in vegetable tissue. One of these was designated as the "V" substance on account of its apparent similarity to the water-soluble vitamine B. This "V" substance was present in laked rabbit blood in quantity sufficient to support the growth of Pfeiffer's bacillus in a dilution of 1:1,000. It could withstand boiling for short periods, but was destroyed by autoclaving and was adsorbed by charcoal. The other substance, which was called "X," was considerably more heat-stable than that first mentioned, as it was not destroyed by autoclaving at 120°C. for 30 minutes. The "X" substance was shown to be present in solutions of crystalline hemoglobin and in plant tissue.

The "V" substance was demonstrated in red blood cells, in watery extracts of red blood cells, bacteria, yeast, tomatoes, peas, and beans. Such extracts added to ordinary broth would give an abundant growth of the Pfeiffer bacillus, provided the seeding was made from a blood broth culture. If, however, a so called "V" culture (containing only the "V," not the "X" substance) was seeded into media containing only the "V" substance, no growth took place in the second transfer, thus showing that the "V" substance alone was not sufficient to maintain growth. A minute amount of autoclaved blood or blood extract added to this "V" culture would, however, suffice to support growth through an indefinite number of transfers, although the same amount of autoclaved blood by itself was incapable of maintaining growth.

Thus it was evident that both of these substances were required for the growth of the Pfeiffer bacillus. It was also shown that this organism was capable of growing in absolutely blood-free media, such as phosphate solutions, containing pieces of sterile, unheated vegetable, such as potato. It was concluded that fresh vegetable tissue contained both the "X" and "V" substances.

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[1] Thjötta, Th., *J. Exp. Med.*, 1921, xxxiii, 763.

[2] Thjötta, Th., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.

[3] Thjötta, Th., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 455.

In working further on the problem, Morgan and Avery[4] have shown that not only the so called hemoglobinophilic bacteria, but also pneumococci and streptococci, are very beneficially affected by the substances in question. The range of hydrogen ion concentration within which growth can be initiated is extended, and, in the case of the pneumococcus, the growth curve is altered by the elimination of the so called "lag period" and by the lengthening of the period in which viable organisms exist in the culture.

The present study was undertaken in the hope of throwing some light upon the nature of the so called "V" substance. Originally Thjötta and Avery considered this substance as similar in nature to the water-soluble vitamine B, since it showed reactions quite similar to those of this substance and could be extracted from sources that were known to be rich in vitamine B. Now it is a well known fact that the lack of vitamine B in certain animals will give rise to a distinct clinical picture. It is likewise known that the animal organism is unable to produce this vitamine for itself, but obtains what it requires from plant tissue. If these substances are identical, it should consequently be possible, by depriving an animal of vitamine B, to demonstrate a relation between the absence of vitamine B in the tissues and the inability of the animal's blood to support growth of Pfeiffer's bacillus.

If the titer of the so called "V" substance is determined during feeding experiments with vitamine B-free diet, this titer should progressively diminish if "V" and vitamine B are identical. The clinical signs of polyneuritis can serve as the indicator of the lack of vitamine B. That all traces of vitamine B have been exhausted by the animal organism, when the clinical signs of the lack of this substance set in, is not known for certain, nor is it known indeed whether the organism is entirely free from this substance at the time when the animals die from polyneuritis. But one may consider it very likely that the last trace of vitamine B will be used by the nervous system of the animal, since that tissue suffers most from the lack of this substance. If the two substances are identical it might be possible to detect a decline in the titer of "V" substance in the blood at or about the time of onset of the clinical symptoms.

[4] Morgan, H. J., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 207.

To test this hypothesis the blood of several normal guinea pigs, rabbits, pigeons, and hens was titrated several times and the fact determined that the usual content of "V" substance was present. The animals were then put on a vitamine B-free diet consisting in part of ordinary food autoclaved at 130°C. for 1 hour, an exposure ample to destroy all the "V" substance present in the food. Most of the food consisted of dry rice. Some fine white bread was given the pigeons and hens and these animals also received in certain instances a small amount of cod liver oil as a source of vitamine A. When necessary the animals were fed with a stomach tube.

At stated periods the blood of these animals was examined as to its "V" content. Blood was taken from the heart in the case of the guinea pigs and rabbits, from the wing vein in that of the pigeons and hens, and laked with 20 parts of distilled water. The titrations were made in ordinary phosphate broth of pH 7.8, and the tubes were seeded with a 24 hour blood broth culture of the Pfeiffer bacillus. Readings were made after 24 hours in the incubator.

Pigeons and hens naturally are much better objects for this investigation than rabbits and guinea pigs, since they tolerate the diet far better and develop distinct symptoms of polyneuritis, whereas guinea pigs and rabbits soon lose appetite, and sicken and die at a relatively early period, without having shown any symptoms resembling those in the pigeons and hens. The results with rabbits proved so indecisive on account of the early death of the animals that they will be omitted.

Experiment 1.—The blood of two guinea pigs titrated for the heat-labile substance "V" showed on two different occasions a titer of 1:800 and 1:1,600 (all titrations were made from 1:200 by halving each dose). On October 11, 1921, the animals were put on the autoclaved diet and rice. During the experiment the following titrations of "V" were made.

Oct.	22.	Titer 1:800.
"	31.	" 1:800.
Nov.	9.	" 1:800.

During this time no typical symptoms of polyneuritis could be noted, but the the animals in the 3rd and 4th weeks were distinctly sick, lacking appetite, losing weight, and keeping very quiet in their cage. On November 15, one of them was found dead, while the other died suddenly during a gavage some days later. Blood taken from this animal immediately after death had a titer of 1:800.

Although this experiment can hardly be said to be conclusive it shows that guinea pigs can live more than 1 month on vitamine B-free diet without a reduction in the titer of the "V" substance as measured by the growth-promoting action of its blood on Pfeiffer's bacillus.

Experiment 2.—The bloods of two pigeons, a male and a female, were titrated as to their content of the "V" substance. While on normal diet the titers were as follows:

Feb. 20, 1923. Titer 1:5,000.
" 26, 1923. " 1:5,000.
" 27, 1923. " 1:3,200.
Mar. 3, 1923. " 1:6,400.

On March 6, the pigeons were put on a diet consisting only of dry rice and water.

Mar. 12, 1923. Titer 1:3,200.
" 19, 1923. " 1:6,400.

From this date the pigeons received in addition to the rice some fine white bread and peas long autoclaved.

Apr. 9, 1923. Titer 1:6,400.

On April 13, the male had a typical attack of polyneuritis, dropping its head backwards, and it was not able to stand on its legs. At 11 o'clock on that day it was given 10 cc. of yeast extract by stomach tube. At 2 o'clock it was found walking about in the cage apparently normal.

On the next morning, it had another attack as marked as that the day before and died. Blood was taken from the heart after death. It showed a titer of 1:6,400.

The female pigeon was fed by stomach tube from April 17 on, as it had completely lost appetite. The nourishment consisted of a solution of sugar, some peptone, and Ringer's solution autoclaved at high temperature, and in addition a small quantity of cod liver oil. The bird had already shown for some days very distinct symptoms of polyneuritis; it tumbled over in the cage, could not take more than a few steps at a time or sit on a perch.

Apr. 17. Titer 1:6,400.
" 19. Died suddenly during the gavage.
Blood titer 1:3,200.

The experiment shows that the clinical signs of vitamine B inanition may be well developed without the titer of "V" dropping appreciably. One of the pigeons died, apparently from lack of vitamine B, without

the titer of "V" decreasing from the normal. The other bird which succumbed during the feeding had unmistakable symptoms of vitamine lack.

Experiment 3.—A hen was put on vitamine-free diet May 10, 1923. Previously the "V" titer had been on several occasions 1:3,200.

The hen appeared to be well for about 1 month, when it began to show signs of polyneuritis. It could walk only with difficulty, showed a tendency to lie down in its cage, and ate only very little. When put on its legs it could not keep its balance. Since it had lost much weight in the last days of the experiment it now received in addition to the rice 10 cc. *per diem* of a mixture of cod liver oil, peptone, glucose, and Ringer's salts. This was given by stomach tube. On June 15, the hen had a typical attack of polyneuritic collapse. The blood showed a "V" titer of 1: 3,200. After the bleeding the hen was given 20 cc. of yeast extract subcutaneously together with 10 cc. of saline solution. Also it was put on a vitamine B-containing diet. The following day it had recovered, and appeared lively. It still showed the polyneuritic manner of walking. On June 18, the rice diet was resumed and a new blood titration was made which again yielded the figure 1:3,200.

In the following weeks the symptoms of the polyneuritis increased steadily and on June 30, when a new titration was made, it could stand on its legs only with difficulty. The blood now titrated 1:3,200.

On July 9 (2 months after the commencement of the experiment), the hen had another attack of polyneuritis. The blood titration showed 1: 3,200. Again 20 cc. of yeast extract were injected subcutaneously. This injection had, however, only a brief and slight effect. On July 11, the hen died.

This experiment shows distinctly that the most serious clinical condition of polyneuritis can develop without any fall in the titer of "V" substance in the blood of the animal. On the contrary, this titer is maintained until the death of the animal.

Experiment 4.—Another strong and active hen was put on vitamine-free diet at the same time as the previous one. The blood titer for both was the same before the experiment—1:3,200. After 1 month on vitamine-free diet (rice) this hen did not show any sign of polyneuritis and was still strong. The titer of "V" was unaltered, 1:3,200. Now the diet was changed to rice, autoclaved potatoes, and peas. The autoclaving was done at 115° C. for 1 hour. To add some vitamine A to this food a small amount of cod liver oil was given in a weak solution of peptone. This was administered by stomach tube once every 2nd or 3rd day.

On this diet the hen thrived well, did not lose weight, was always very active, never showed signs of weakness or a polyneuritic manner of walking. It never

had any food which was not either autoclaved or consisted of rice. Several titrations were made during the summer and autumn of 1923, always with the same result; namely, a titer of 1:3,200. In January, 1924, the hen had been kept for 7 months on this diet without showing any signs of polyneuritis and on January 20, 1924, it was put back on a diet exclusively of rice.

Titrations of "V" showed:

Mar. 13. Titer 1:3,200.
Apr. 15. " 1:3,200.

During the last week of April the hen began to lose its appetite and did not look well. On April 29, it was found lying in the cage. It showed now a typical picture of polyneuritis. The blood titrated 1:3,200. No yeast extract was given, and on the next morning the hen was found lying on the floor of its cage unable to raise the head. A new titration yielded the figure 1: 3,200. 10 cc. of yeast extract were given by stomach tube and 10 cc. subcutaneously. Later the fowl was given by gavage scrapings of fresh potatoes and this was repeated the next day. Within 3 days the animal seemed quite recovered and in a few days more it looked and behaved like a normal hen.

This experiment shows that a hen living on autoclaved food for 7 months failed to develop signs of polyneuritis, whereas 3 months after a change to a diet exclusively of rice it showed symptoms in a marked form. Neither of these diets had any effect on the titer of "V" in the fowl's blood.

The heating of the food in this experiment cannot, as its result shows, have been sufficient to destroy all of the vitamine B, but must have left enough of that substance to ward off polyneuritis. The fact is now well known that the "V" substance does not stand heating at 120° for half an hour. How high a temperature "V" will resist has not been determined. It is therefore a matter of interest to ascertain whether the temperature employed in Experiment 4 is sufficient to destroy the "V." In Experiment 5 the thermostability of "V" is tested.

Experiment 5.—Yeast extract produced after the method of Thjötta and Avery was autoclaved for half an hour at a temperature of 105°, 110°, and 115°C. After having cooled down, the three specimens were added to ordinary broth in concentration of 10 per cent. These media, as well as the controls, were inoculated with 1 drop of a 24 hour culture of the Pfeiffer bacillus in blood broth. The result was as follows:

	Yeast extract autoclaved for ½ hr. at:			Yeast extract not autoclaved.	No yeast extract.
	105°C.	110°C.	115°C.		
Growth	−	−	−	++	−

This experiment shows that the growth-promoting substance "V" is destroyed at 105°C. On the other hand, Experiment 4 showed that food of a sort in which vitamine B is present did not lose its property of warding off polyneuritis after being autoclaved for 1 hour at 115°C. This is in full accordance with experiments carried out by previous investigators. Thus Holst[5] found that heating of dried peas and barley at 115°C. for 30 minutes did not destroy their antipolyneuritic power. Chick and Hume[6] found that the antipolyneuritic power of yeast extract (containing 60 per cent of water) was only slightly diminished after heating at 122°C. for 1 hour.

There is then a distinct difference in the thermostability of the growth-promoting substance "V" and the vitamine B. This difference points to a difference in the nature of these substances, although the possibility cannot be denied that the antipolyneuritic action in the animal organism and the growth-promoting action in the bacterial culture are but two functions of the same substance, one of which is more heat-stable than the other. This is, however, a mere theoretical consideration, and we will at the present time have to accept the difference in thermostability as an indication of a difference in the nature of the two substances.

SUMMARY.

It has been shown in several experiments that the heat-labile, growth-promoting factor "V" found in the blood of animals does not diminish or disappear when the animals are kept on vitamine B-free diet. The titer of the "V" substance present in the blood of normal animals is undiminished in animals showing severe signs of polyneuritis even to the time of their death.

[5] Holst, A., *J. Hyg.*, 1907, vii, 619.

[6] Chick, H., and Hume, E. M., *Proc. Roy. Soc. London, Series B*, 1917–19, xc, 60.

The antipolyneuritic factor of vegetable food, designated as vitamine B, is found to be considerably more heat-stable than is the "V" substance concerned in promoting the growth of Pfeiffer's bacillus.

CONCLUSIONS.

The heat-labile, growth-promoting factor "V" necessary for the cultivation of the bacillus of Pfeiffer is not identical with vitamine B.

In the blood of animals that have been fed for several weeks on a diet free of vitamine B, the growth-promoting factor "V" is found in the same strength as in animals fed upon normal diet. Consequently it must be synthetized by the animal organism.

THE RELATION OF DOSE OF BACTERIA TO GROUP INFECTION.

By ARTHUR L. BLOOMFIELD, M.D., and AUGUSTUS R. FELTY, M.D.

(*From the Biological Division of the Medical Clinic of the Johns Hopkins University and Hospital, Baltimore.*)

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It is recognized that there exist in every community a considerable number of healthy people who harbor in the upper respiratory tract pathogenic microorganisms such as streptococci, diphtheria bacilli, and meningococci. While these "carriers" are to be regarded as storehouses of bacteria which may ultimately lead to the production of disease in other people, it has become clear that they are for the most part quite innocuous under the average conditions of human association, and that some special combination of circumstances is necessary before secondary carriers or instances of clinical disease will develop. The exact analysis of the factors which determine such spread of infection is not only of interest from a biological standpoint, but is also of the utmost practical importance in the standardization of public health measures.

On the basis of clinical observations and test-tube experiments it was suggested in a previous paper (1) that the dose of bacteria and the degree of their vegetative activity were at least two factors which were significant in determining whether or not infection will develop in a susceptible host. The matter was discussed in detail at that time and the present communication deals with animal experiments which extend and confirm the conclusions previously reached, especially with reference to the relation of dose to group infection.

It has been definitely established in experimental work that dosage bears an important relation to infection. In general, the larger the dose the greater is the likelihood that infection will take place, although if one deals with a population which is highly susceptible to a

certain organism, even a minute number of the bacteria will regularly produce disease, as for example in the case of mice inoculated with fixed type pneumococci. But with a population which on the whole is highly resistant to a given infectious agent the situation is less clearly understood. Webster (2), for example, in another connection has noted that an increase of dose of one thousand-fold increases the mortality from mouse typhoid infection in mice only from 40 per cent to 75 per cent, and one of Topley's experiments (3) suggests a similar state of affairs. It is evident therefore that under certain conditions no direct proportion exists between size of dose and likelihood of infection.

Clinical observations on the effect of crowding, which have been discussed in detail in the previous paper, suggest that in the presence of carriers of certain varieties of pathogenic bacteria a very high degree of intimate contact is necessary before spread of infection takes place at all; that as the degree of contact is reduced there is not a gradual falling off in the number of secondary infections but at a certain point they diminish with great abruptness to a figure approximating zero. In other words, it seems probable that people who are relatively resistant to a certain organism must receive a dose greater than some critical value before any infection will result, and that smaller doses, even if pathogenic bacteria are actually received, for example into the upper air passages, will be quite inadequate, other things being equal, to colonize, to invade, or to produce disease. Such an idea explains the usual harmlessness of the diphtheria bacillus, streptococcus, or meningococcus carrier. His associates acquire the organisms, to be sure, but under ordinary conditions of contact only a subcritical dose in the sense indicated above.

In the present experiments an attempt was made to analyze *in vivo* the relation between dosage and resulting group infection.

Methods.

It seemed best to choose an experimental host whose susceptibility to the organism employed was of the same general degree as that which exists in man towards the common pathogens of respiratory

infection. White mice and hemolytic streptococcus were finally selected as fulfilling these conditions.

The animals all came from the same breeder, and while they were of about the same size, no attempt was made to have exact uniformity of age, weight, or sex. The usual methods of feeding and caging were used and there was practically no trouble from spontaneous infection or cannibalism. The hemolytic streptococci used in most of the experiments were originally isolated from cases of acute tonsillitis and were of the beta type (Brown). They were shown to be colony-pure by numerous platings, and were preserved on human blood agar from which transfers to broth were made for the experiments. Broth cultures were centrifuged and the growth resuspended before inoculation. In this way confusion from introduction of culture fluid was avoided. Careful counts of the culture by the plate method were always made just before the experiment was done.

All inoculations were made intraperitoneally and the various doses of bacteria were in each case suspended in 0.5 cc. of broth or salt solution so that the volume of fluid injected was always uniform.

At 24 hour intervals after inoculation small amounts of peritoneal exudate were withdrawn from the mouse by capillary pipette. A standard loop of the fluid was cultured in blood agar and smears were studied in respect to cellular reaction, number of bacteria, phagocytosis, etc. Autopsies were made as soon as possible after death and the heart's blood, peritoneum, and pleura were cultured. Death was regarded as specific if hemolytic streptococci were recovered at autopsy from one or more situations in large numbers and if other bacteria were absent or obtained only in small numbers.

Several types of reaction to the inoculations were observed and they may now be appropriately described.

Course of Streptococcus Infection in the Mouse Following Intraperitoneal Inoculation.

1. Primary Specific Death.—There is a prompt and rapid growth of the bacteria and the animal dies in 24 hours or less. No gross lesions are found but innumerable organisms are present in peritoneum and heart's blood, and peritoneal exudate shows only a few fragmented leucocytes. There are variable degrees of phagocytosis. The animal is apparently overwhelmed by the infection and offers little resistance as evidenced by cellular reaction.

2. Delayed Primary Specific Death.—The animal at first appears to overcome the infection. After 24 hours the peritoneal exudate shows great numbers of leucocytes with active phagocytosis and relatively few bacteria. But within 48 hours death occurs with similar findings to those in Group 1.

3. Secondary Specific Death.—Peritoneal puncture at 24 hours shows profuse leucocytic exudate and the streptococci have disappeared. The animal may appear well for several days but eventually dies and at autopsy shows but little peritoneal reaction and few organisms in the peritoneum, but a general infection is present with positive blood culture and local lesions in lungs, pleuræ, and viscera. Secondary invasion by other bacteria may take place in this group.

4. Survival.—In those animals which survive, the peritoneum at 24 hours almost invariably shows abundant exudate with many leucocytes but no bacteria either in smear or on culture.

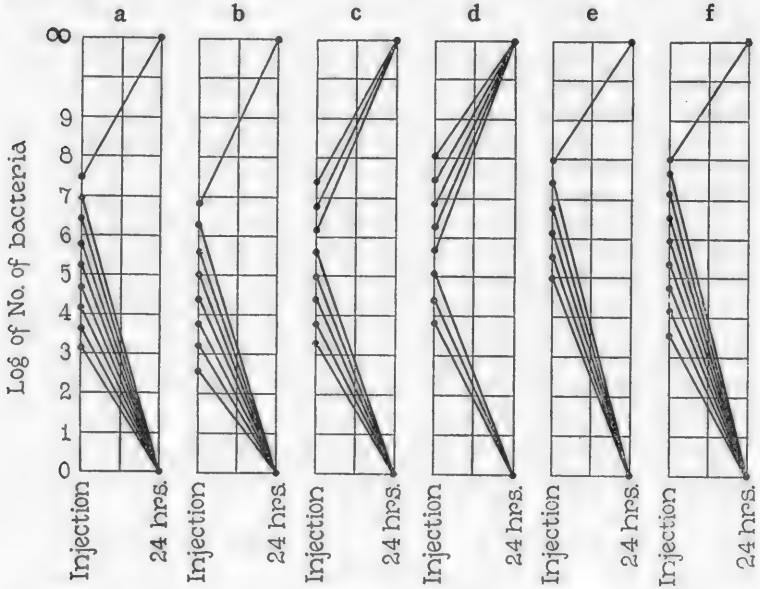

CHART 1, *a* to *f*. Graph of Experiments 1 to 6. (*a*) Experiment 1; (*b*) Experiment 2; (*c*) Experiment 3; (*d*) Experiment 4; (*e*) Experiment 5; (*f*) Experiment 6. Each line represents one animal.

All the data are shown in the protocols, but a consideration of these types of reaction indicates the necessity of selecting a definite criterion for comparison of the effects of various doses of bacteria. In the present work we were concerned strictly with the relation of *initiation* of infection to dose. It seemed logical, therefore, to be guided by the findings on peritoneal puncture 24 hours after inoculation, regardless of subsequent death or survival of the animal.

If the 24 hour smear showed innumerable streptococci it was clear that infection had been initiated. If no bacteria were seen infection was not considered as having taken place even if it turned out later that a few bacteria had actually survived and had gone on to produce

Experiments 1 to 6.

Mouse No.	Dose.	No. of bacteria injected.	Result.	24 hr. peritoneal puncture.		
				White blood corpuscles per field.	Phagocytosis.	Streptococci.
Experiment 1. Nine mice. 6 hr. broth culture of Streptococcus 205 injected in decreasing amounts.						
	cc.					
1	1/4	37,500,000	D. 36 hrs.	About 100.	0	∞ *
2	1/16	9,350,000	S. 7 days.	" 100.	0	0
3	1/64	2,500,000	" 7 "	" 100.	0	0
4	1/250	600,000	" 7 "	" 100.	0	0
5	1/1,000	153,000	" 7 "	" 100.	0	0
6	1/4,000	38,400	" 7 "	" 100.	0	0
7	1/16,000	9,600	" 7 "	" 100.	0	0
8	1/64,000	2,400	" 7 "	" 100.	0	0
9	1/250,000	600	" 7 "	" 100.	0	0
Experiment 2. Eight mice. 6 hr. broth culture of Strain R injected in decreasing amounts.						
1	1	7,600,000	D. 12 hrs.	Few.	0	∞†
2; 3; 4; 5; 6; 7; 8	1,4; 1/16; 1/64; 1/250; 1/1,000; 1/4,000; 1/16,000	1,900,000; 475,000; 118,700; 29,700; 7,400; 1,800; 450	S. 7 days.	About 200.	0	0
Experiment 3. Eight mice. 5 hr. ascites broth culture of Streptococcus R injected in decreasing amounts.						
1	2	28,000,000	D. 9 hrs.	20	+	∞†
2	1/2	7,000,000	" 18 "	10	0	∞
3	1/8	1,750,000	" 18 "	20	0	∞
4; 5; 6; 7; 8	1/32; 1/128; 1/500; 1/2,000; 1/8,000	437,000; 109,000; 27,000; 6,700; 1,670	S. 6 days.	About 100.	0	0

* ∞ = innumerable.

† Postmortem.

Mouse No.	Dose.	No. of bacteria injected.	Result.	24 hr. peritoneal puncture. White blood corpuscles per field.	Phagocytosis.	Streptococci.
Experiment 4. Eight Mice. 5 hr. ascites broth culture of Streptococcus R injected in decreasing amounts.						
	cc.					
1	2	140,000,000	D. 16 hrs.	Few.	0	∞†
2	1/2	35,000,000	" 16 "	"	0	∞†
3	1/8	8,750,000	S. 3 days.	"	0	∞
4	1/32	2,180,000	D. 18 hrs.	None.	0	∞†
5	1/128	540,000	" 16 "	Few.	0	∞†
6; 7; 8	1/500; 1/2,000; 1/8,000	135,000; 33,700; 8,400	S.	About 100.	0	0
Experiment 5. Five mice. 3 day ascites broth culture of Streptococcus R injected in decreasing amounts.						
1		102,000,000	D. 22 hrs.	Few.	0	∞†
2		25,500,000	S. 6 days.	About 100.	0	0
3		6,300,000	" 6 "	" 100.	0	0
4		1,570,000	" 6 "	" 100.	0	0
5		390,000	" 6 "	" 100.	0	0
Experiment 6. Nine mice. 3 day broth culture of Streptococcus 205 injected in decreasing amounts.						
1		112,000,000	D. 36 hrs.	About 200.	+	∞
2		56,000,000	S. 7 days.	" 200.	0	0
3		14,000,000	" 7 "	" 200.	0	0
4		3,580,000	" 7 "	" 200.	0	0
5		890,000	" 7 "	" 200.	0	0
6		224,000	" 7 "	" 200.	0	0
7		56,000	" 7 "	" 200.	0	0
8		14,000	" 7 "	" 200.	0	0
9		3,500	" 7 "	" 200.	0	0

a late reaction. Only rarely was there a border line finding; at 24 hours the peritoneum was either free of organisms (smear) or showed them in overwhelming numbers.

EXPERIMENTS.

It seemed advisable before studying group infection to discover the effect of decreasing doses of bacteria injected into single series of mice.

Discussion of Experiments 1 to 6.—A consideration of the protocols shows a state of affairs strikingly similar to that brought out by the previously reported test-tube experiments. It is seen that as the dose of bacteria is decreased by one-half or by three-fourths there is a critical point below which infection was not initiated. Whereas a certain dose led to tremendous growth in the mouse peritoneum in 24 hours, the succeeding dose resulted not in a slower multiplication but in a total failure to initiate infection. The results are shown graphically in Chart 1. It will appear later that occasionally there is a border line reaction, but in the vast majority of cases either prompt infection or complete lack of initiation occurs.

The next experiments deal with the effects of various doses of bacteria injected into *groups* of mice. The attempt was made roughly to parallel a population of fairly uniform resistance to streptococcus exposed in varying degree to the risk of infection.

Experiment 7 A.—Twenty mice. Five cages of four mice each. 23 hour meat infusion broth (pH 7.4) culture of Streptococcus 205 centrifuged, resuspended in broth, and injected intraperitoneally in decreasing concentration. Count of culture before inoculation about 200 million per cc.

It is immediately apparent that mortality rates in the various groups were not proportional to the dose of culture. In a general way death occurred more frequently and more rapidly with the larger inoculations, but No. 3, receiving a maximum dose, survived 6 days and died a non-specific death, and Nos. 19 and 20 died whereas Nos. 9 and 12, receiving four times the dose, survived. These variations are evidently to be explained by differences of resistance of individual mice, and the point to be stressed is that, regardless of dose, the mortality was high in each group.

Considered from the point of view of immediate initiation of infection the results are even more striking. The 24 hour peritoneal punctures show that reduction of dose from 1.0 cc. to 0.5 cc. led to an abrupt change in reaction. With doses of 0.5 cc. or less there was no evidence of peritoneal multiplication in 24 hours, and all the deaths which later occurred were secondary in type. With doses of 1 cc. or above, on the other hand, primary infection occurred in six of eight cases.

Experi

Mouse No.	Dose of culture.	24 hr. peritoneal puncture.					48 hr. peritoneal puncture.				
		Smear.				Culture.	Smear.				Cultu
		White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	No. of hemolytic streptococci per loop.	White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	No. of hemolytic streptococci per loop.
	cc.										
1	2	50–100	+	+++	Good.	2,000,000					
2	2	3–400	++	Few.	"	150,000	25	–	+++	Good.	8
3	2	3–400	+	"	"	2,000	25	–	0	–	
4	2	20	++	++++	"	∞					
5	1	20	+	++++	"	∞					
6	1	200	+	++	Poor.	800,000					
7	1	200	+	+	"	∞					
8	1	200	+	+	"	∞					
9	0.5	1–200	+	Few (intracellular).	Good.	280	8	–	0	–	
10	0.5	1–200	–	0	–	0	25–50	–	0	–	
11	0.5	1–200	–	0	–	0	100	+	Few.	Good.	3,0
12	0.5	1–200	–	0	–	0	50	+	0	–	
13	0.25	1–200	–	0	–	0	100	–	0	–	
14	0.25	1–200	–	0	–	0	10	–	0	–	
15	0.25	1–200	–	0	–	0	10	–	0	–	
16	0.25	1–200	–	0	–	0	10	–	0	–	
17	0.125	1–200	–	0	–	0	100	–	0	–	
18	0.125	1–200	–	0	–	0	100	–	0	–	
19	0.125	1–200	–	0	–	200	20	–	0	–	
20	0.125	1–200	–	0	–	0	20	–	0	–	

ment 7A.

Outcome.	Postmortem peritoneal puncture.							Lesions.
	Smear.				Culture.			
	White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	Peritoneum.	Heart's blood.	Pleura.	
D. 24 hrs.					∞			No gross lesions.
" 3 days.	Few.	0	∞	Good.	∞	0		
" 6 "	25	—	No streptococci.	—	No streptococci. Mixed culture.	0		
" 24 hrs.					∞			
" 24 "					∞			
" 40 "	100	0	+++	Good.	∞	+		
" 34 "					∞			
" 34 "	20	0	++++	"	∞	+		
S.								
D. 4 days.	75	0	Few.	Fair.			+++	Purulent pleurisy.
" 3 "	1–200	0	+	Good.	∞	+		
S.								
D. 3 days.	100	0	0	—	0	0		No lesions.
" 3 "	Few.	0	Few.	Good.	200 per loop.	+		
" 5 "	25	—	None seen.	—	15,000 per loop.	++	+++	Pneumonia. Empyema.
S.								
n								
n								
D. 3 days.	1–200	0	+++	Good.	∞	+		
" 6 "	25		+ Also Gram-positive bacilli.	"	8,000 per loop.	+++		No gross lesions.

Exper

Mouse No.	Dose of culture.	24 hr. peritoneal puncture. Smear. White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	24 hr. peritoneal puncture. Culture. No. of hemolytic streptococci per loop.	48 hr. peritoneal puncture. Smear. White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	48 hr. peritoneal puncture. Culture. No. of hemolytic streptococci per loop.
	cc.										
1	1	25	+	++++	Good.	∞					
2	1	50	+	++++	"	∞					
3	1	25	++	++++	"	∞					
4	1	25 Fragmented.	±	++++	"	∞	Few. Fragmented.	0	+++	Good.	∞
5	0.5	200	+	Very Few.	"	10,000	200	+	None extracellular.		
6	0.5	100	±	+++	"	∞					
7	0.5	50	+	+	"	20,000	100	0	0		
8	0.5	50	+	++++	"	∞					
9	0.25	100	±	++++	"	∞	Few. Fragmented.	0	++	"	100,00
10	0.25	10	−	++	"	∞					
11	0.25	100	+	+++	"	280,000	100	++	None extracellular.		5
12	0.25	10	0	++++	"	∞					
13	0.125	200	0	0	−	200	20	0	0	−	
14	0.125	200	0	0	−	0	50	0	0	−	
15	0.125	200	0	+	Good.	30,000	25	++	+++	Good.	120,00
16	0.125	100	0	+++	"	∞					
17	0.062	25	0	0	−	0	50	0	0	−	
18	0.062	0	0	++++	Good.	∞					
19	0.062	25	0	0	−	0	50	0	0	−	
20	0.062	25	0	++++	Good.	∞					

ment 7B.

Outcome.	Postmortem peritoneal puncture.							Lesions.
	Smear.				Culture.			
	White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	Peritoneum.	Heart's blood.	Pleura.	
D. 36 hrs.	20	+	∞	Good.	∞	+		No gross lesions.
" 37 "	25	+	+++	"	∞	+		Pneumonia.
" 27 "	Few.	0	∞	"	∞	+		
" 3 days.	0	0	++	"	Many.	++		"
" 4 "	Few. Fragmented.	+	++	"	∞	+		" plus pleurisy.
" 48 hrs.	Few.	0	∞	"	∞	+		
" 5 days.	" Fragmented.	0	++	"				No gross lesions.
" 36 hrs.	20 Fragmented.	+	∞	"	∞	+	+	Pneumonia.
" 3 days.	Few. Fragmented.	0	++	"	∞	++		
" 36 hrs.	Few. Fragmented.	0	∞	"	∞	+		
" 5 days.	Few. Fragmented.	0	++	"				No gross lesions.
" 36 hrs.	Few. Fragmented.	0	∞	"	∞	+		
" 4 days.	10	0	+++	"	∞	+		Pneumonia.
" 4 "	25	0	0	—	0	+		No gross lesions.
" 3 "	10	0	Few.	Good.	Many.	+		
" 36 hrs.	100	+	++	"	∞	+		Pneumonia.
S. D. 36 hrs.	0	—	∞	"	Many.	++		
S. D. 36 hrs.	Few.	—	++	"	∞	++		

Experiment 7 C.

Mouse No.	Dose of culture.	Outcome.	Postmortem peritoneal puncture.							Lesions.
			Smear.				Culture.			
			White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	Peritoneum.	Heart's blood.	Pleura.	
	cc.									
1	1.0	D. 22 hrs.	Few.	0	∞	Good.	∞	+		No gross lesions.
2	0.125	" 19 "	1	++	∞	"	∞			" " "
3	0.125	" 20 "	25	++	∞	"				" " "
4	0.125	" 21 "	Few.	++	∞	"	∞			" " "
5	0.125	" 23 "	"	0	∞	"	∞			" " "
6	0.125	" 3 days.	"	+	∞	"	∞			
7	0.062	" 24 hrs.	" Fragmented.	0	∞	"	∞			" " "
8	0.062	" 36 "	25 Fragmented.	0	∞	"	∞			" " "
9	0.062	" 18 "	Few.	++	∞	"	∞			" " "
10	0.062	" 19 "	25	+	∞	"	∞			" " "
11	0.062	" 36 "	Few. Fragmented.	0	∞	"	∞			" " "
12	0.031	" 24 "	Few. Fragmented.	0	∞	"	∞			
13	0.031	" 27 "	Few.	0	∞	"	∞			
14	0.031	" 7 days.	0	0	∞	"	∞			
15	0.031	S.								
16	0.031	D. 23 hrs.	25	+	∞	"	∞			

17	0.015	D. 27 hrs.	Few. Fragmented.	0	∞	"	∞			
18	0.015	" 24 "	Few. Fragmented.	0	∞	"	∞			
19	0.015	S.								
20	0.015	D. 36 hrs.	200	0	Few.	"	Many.	+	+++	Pleurisy.
21	0.015	" 36 "	Few. Fragmented.	0	∞	"	∞			
22	0.0078	" 18 "	Few.	++	∞	"	∞			No gross lesions.
23	0.0078	" 24 "	0	0	∞	"	∞			" " "
24	0.0078	" 36 "	Few. Fragmented.	0	∞	"	∞			" " "
25	0.0078	S.								
26	0.0078	"								
27	0.0039	D. 26 hrs.	Few.	0	+++	"	∞			
28	0.0039	" 36 "	"	0	++	"	∞			
29	0.0039	" 36 "	"	0	∞	"	∞			
30	0.0039	" 3 days.	" Fragmented.	0	∞	"	∞			
31	0.0039	" 3 "	Few. Fragmented.	0	∞	"	∞			
32; 33; 34; 35	0.001	S.	Many.	0	0	—	0			
36; 37; 38; 39	0.0001	"	"	0	0	—	0			
40; 41; 42; 43	0.00001	"	"	0	0	—	0			

Experiment 7 B.—Twenty mice. Five cages of four mice each. 23 hour meat infusion broth (pH 7.4) culture of Streptococcus 205 centrifuged, resuspended in broth, and injected intraperitoneally in decreasing concentration. Count of culture before inoculation about 200 million per cc.

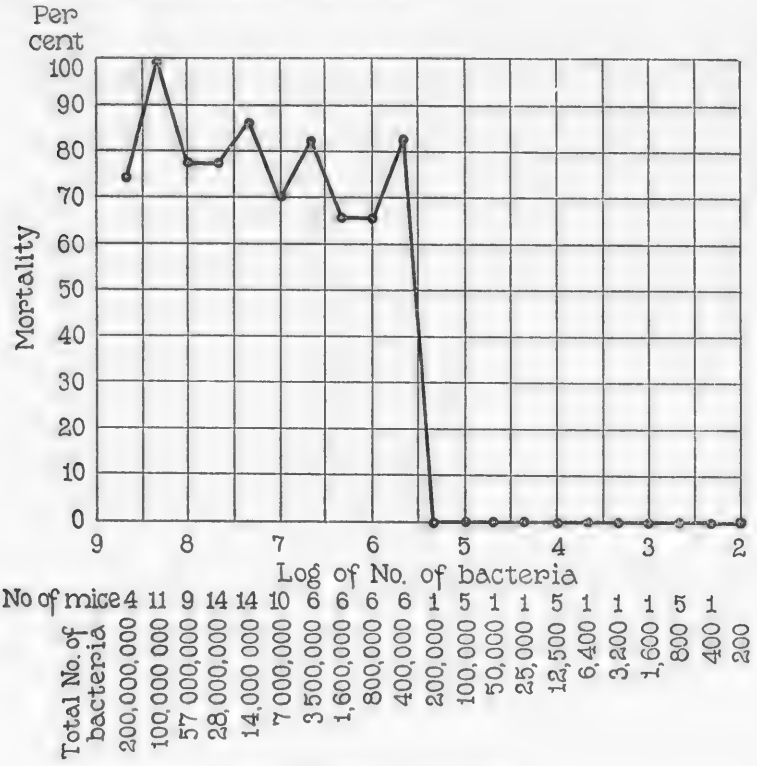

CHART 2. Graph of Experiment 7.

In this experiment all the mice except two in the last group eventually died. But within each group great variations in reaction are seen as indicated by the peritoneal punctures and the time of survival. Thus Nos. 18 and 20 died in 36 hours with an immediate overwhelming reaction, while No. 7 which received eight times the dose nearly overcame the infection and survived for 5 days.

Experiment 7 C.—Forty-three mice. One cage with one mouse, six cages with five mice, three cages with four mice. 23 hour meat infusion broth (pH 7.4) culture of Streptococcus 205 centrifuged, resuspended in broth, and injected intraperitoneally in decreasing concentrations. Count of culture before inoculation about 200 million per cc.

In this experiment a wider range of dosage was used. Events among Mice 1 to 31 correspond with those brought out in Experiments 7 A and 7 B. But when the dose was decreased from 0.0039 cc. to 0.001 cc. the mortality abruptly fell to zero.

In Chart 2 the results of Experiments 7 A, 7 B, and 7 C, together with certain other similar observations, are graphically reproduced. The logs of the numbers of bacteria are plotted as abscissæ and the percentage mortalities for all the groups receiving a uniform dose are plotted as ordinates. The total number of bacteria and the total number of animals in each group are also indicated. Conditions as regards the bacteria were as uniform as possible except for dosage, but the curve must receive a liberal interpretation inasmuch as it represents the composite result of several experiments. However, it offers a fair analogy to conditions as they perhaps exist in a human community containing many streptococcus carriers and many susceptibles. It is seen that decrease in dosage does not run parallel with decrease in mortality. In the zone above 400,000 bacterial infection is likely to occur to some extent regardless of dose; in the zone below 200,000 infection never took place.

In the next experiment similar methods were used but an attempt was made to increase "resistance" of the mice as a whole by suspending the bacteria in salt solution instead of in broth.

Experiment 8.—Forty-five mice. Eleven cages of four mice, one cage of one mouse. 23 hour meat infusion broth (ph 7.4) culture of Streptococcus 205 centrifuged and resuspended in salt solution. Count of culture before injection about 200 million per cc.

The results as shown in the protocol and in Chart 3 correspond closely with those of Experiment 7 save that the critical point above which infection occurred corresponded to a larger dose of bacteria.

Experiment 9.—Forty-six mice in groups of five or six received decreasing amounts of a 6 hour ascites broth culture of Streptococcus R. The results are shown in the protocol and in Chart 4 and correspond essentially with those previously described.

Experiment 8.

Mouse No.	Dose of culture.	24 hr. peritoneal puncture. Smear. White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	24 hr. peritoneal puncture. Culture. No. of streptococci per loop.	Outcome	Postmortem peritoneal puncture. Smear. White blood corpuscles per field.	Phagocytosis.	Streptococci.	Staining.	Postmortem peritoneal puncture. Culture. Peritoneum.	Heart's blood.	Pleura.	Lesions.
	cc.														
1	8.0	25	++	++++	Good.	∞	D. 36 hrs.	20	0	∞	Good.	∞	+		
2	8.0	25	++	++++	"	∞	" 29 "	25	+	∞	"	∞	+		Fibrinous peritonitis.
3	8.0	100	++	++++	"	∞	" 48 "	25	++	∞	"	∞	+		
4	8.0						" 23 "	25	+	∞	"	∞	+		
5	4.0	25	+	++++	"	∞	" 36 "	20	+	∞	"	∞	+		
6	4.0	25	+	++	"	∞	" 29 "	20	+	∞	"	∞	+		Peritonitis.
7	4.0	100	+	Few.	"	1,500	" 5 days.	0	−	+	"	Few.	++		"
8	4.0	100	+	++	"	∞	" 50 hrs.	75	++	∞	"	∞	+		Pericarditis.
9	2.0	100	+	+	"	7,000	" 4 days.	50	0	Few.	"	Few.	+		No gross lesions.
10	2.0	25	0	0	−	500	" 3 "	100	0	"	"	"	++	++	Pericarditis. Empyema.
11	2.0	25	0	0	−	0	S.								
12	2.0	100	0	0	−	0	D. 5 days.	Few.	0	0	−	"	++	+++	Empyema.

13	1.0	1–200	0	+	Good.	4,500	S.								
14	1.0	1–200	0	0	—	250	D. 48 hrs.	5	0	+++	Good.	∞	0		No gross lesions.
15	1.0	1–200	0	0	—	0	S.								
16	1.0	1–200	0	0	—	0	"								
17	0.25	1–200	—	0	—	15	"								
18	0.25	1–200	—	0	—	0	"								
19	0.25	25	—	0	—	0	"								
20	0.25	50	—	0	—	0	"								
21	0.125	25	—	0	—	0	"								
22; 23; 24; 25	0.0625	1–200	—	0	—		"								
26; 27; 28; 29	0.0156	1–200	—	0	—		"								
30; 31; 32; 33	0.0039	100	—	0	—		"								
34; 35; 36; 37	0.0001	100	—	0	—		"								
38; 39; 40; 41	0.000025	100	—	0	—		"								
42; 43; 44; 45	0.000012	100	—	0	—		"								

Mouse No.	No. of bacteria injected.	Outcome.	24 hr. peritoneal puncture.			
			White blood corpuscles per field.	Phagocytosis.	Streptococci.	Stain
1; 2; 3; 4; 5; 6	14,000,000	D. 16 hrs.				
7; 8; 9; 10; 11; 12	4,600,000	" 16 "				
13; 14; 15; 16	1,500,000	" 16 "				
17	1,500,000	" 19 "				
18	500,000	" 16 "				
19	500,000	" 36 "	100	0	∞	Good
20	500,000	S. 7 days.	25	0	0	-
21	500,000	D. 30 hrs.	Few.	0	++++	Good
22	500,000	" 24 "	10	0	∞	"
23	500,000	S. 7 days.	200	0	0	-
24	175,000	D. 7 "	200	++	None extracellular.	-
25	175,000	" 7 "	200	0	0	-
26	175,000	" 4 "	100	0	0	-
27	175,000	" 2 "	25	0	++	Good
28	175,000	" 6 "	1–200	0	0	-
29	175,000	" 23 hrs.	Few.	0	∞	Good
30	57,000	" 23 "	"	0	∞	"
31	57,000	" 6 days.	200	+	None extracellular.	-
32	57,000	" 7 "	200	0	0	-
33	57,000	S. 7 "	200	0	0	-
34	57,000	D. 6 "	200	0	0	-
35	57,000	" 5 "	200	0	0	-
36; 37; 38; 39	19,000	S. 7 "	200	0	0	-
40	19,000	D. 7 "	200	0	0	-
41	19,000	S. 7 "	200	0	0	-
42; 43; 44; 45; 46	6,500	" 7 "	200	0	0	-

9.

Postmortem puncture.				Lesions.	Remarks.
ite cor-les ield.	Phagocytosis.	Streptococci.	Staining.		
.	0	∞	Good.	No gross lesions.	
	0	∞	"	" " "	
	0	∞	"	" " "	
	0	∞	"	" " "	
	Slight.	∞	"	" " "	
	0	∞	"	" " "	
	0	∞	"	" " "	
	0	∞	"	Peritonitis. Empyema. Pneumonia.	
-	—	Many.			Culture of heart's blood ++.
.	0	∞	"	No gross lesions.	
	0	∞	"		
)	0	∞	"		
				" " "	
				" " "	
pecimens lost—probably specific death.					
				Abscess in groin.	
.	0	Few streptococci. Many other bacteria.		Peritonitis.	Possibly non-specific death.
	0	∞	Good		
	0	∞	"	"	

Discussion of Experiments 7 to 9.—The essential point which has been brought out is that with a population (mice) whose resistance as a whole to a certain organism is great there exists a critical zone of dosage above which a high incidence of infection follows, and below which infection does not occur at all, despite the injection of large numbers of viable organisms. The largest doses which the animals uniformly survived would correspond roughly to 10 billion streptococci for an average human adult. The experiments lend support, there-

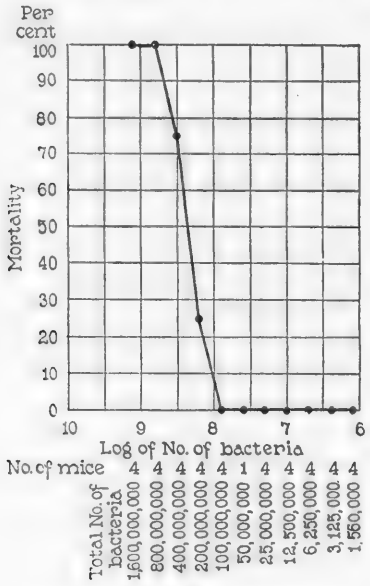

CHART 3. Graph of Experiment 8.

fore, to the ideas previously advanced on clinical grounds, and on the basis of test-tube experiments, and may explain why the chronic respiratory tract carrier of pathogenic bacteria is so often innocuous. Probably the dose received by his associates under ordinary conditions is in the subcritical zone. The experiments also explain epidemiological phenomena such as those studied by Glover with meningitis, when it was found that a difference of $1\frac{1}{2}$ feet in the spacing of beds in a barrack determined whether or not spread from carriers and consequent clinical cases would occur.

A few experiments were done with other varieties of bacteria to test the constancy of the principle which held with streptococcus.

Staphylococcus aureus.

A strain recently isolated from a case of staphylococcus septicemia was used. It was distinctly pathogenic for mice on intraperitoneal injection, although larger doses were necessary in order to kill than with the strains of streptococcus previously employed. Furthermore,

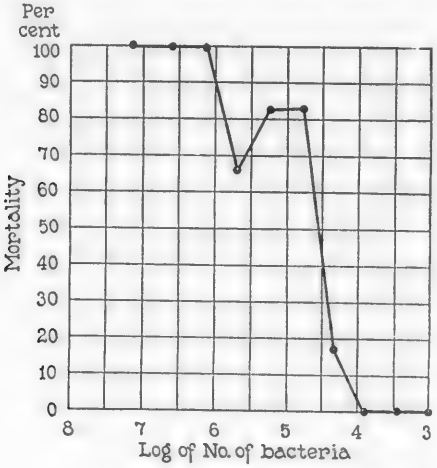

CHART 4. Graph of Experiment 9.

the type of peritoneal reaction was different. Even in animals which died within 18 hours after injection no overwhelming growth of bacteria was found in the peritoneum, and there were always many leucocytes which showed evidence of active phagocytosis. However, results of group infection were similar to those which have already been described. Chart 5 shows the results of an experiment with *Staphylococcus aureus.*

Bacillus coli.

A stock strain was found highly satisfactory. When injected into a series of mice in decreasing doses very clean-cut effects were ob-

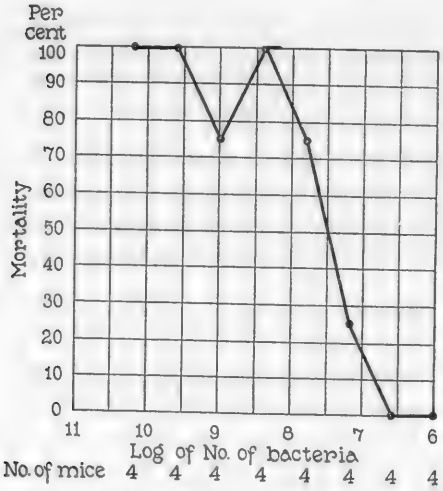

CHART 5. *Staphylococcus aureus.*

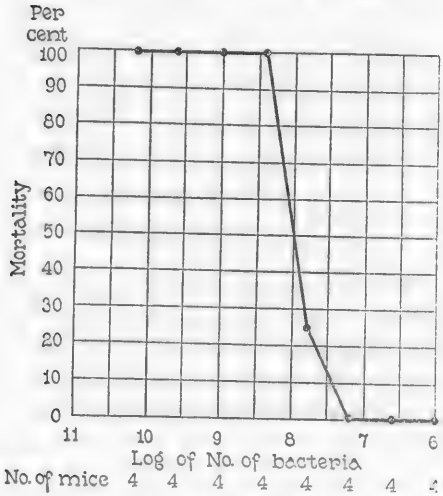

CHART 6. *B. coli.*

tained. The mice either died within 24 hours or survived, and there were no late deaths. In fatal cases the peritoneal smear showed no cells but masses of colon bacilli; in the survivors there was abundant exudate without bacteria. A group infection experiment (Chart 6) shows a critical point below which even large doses of colon bacilli uniformly fail to infect.

In summary, then, the colon and staphylococcus experiments support the results obtained with streptococcus.

SUMMARY AND CONCLUSIONS.

A study has been made of the peritoneal reaction in mice after injection of different bacteria in graded dose. Organisms were employed to which the animals were fairly resistant. Under these conditions it was found that as the dosage was decreased a more or less critical point was reached below which infection failed to be initiated.

The possible clinical bearings of this observation are discussed.

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THE RELATION OF VEGETATIVE ACTIVITY OF BACTERIA TO PATHOGENICITY.

By AUGUSTUS R. FELTY, M.D., and ARTHUR L. BLOOMFIELD, M.D.

(*From the Biological Division of the Medical Clinic of the Johns Hopkins Hospital and University, Baltimore.*)

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In a consideration of certain factors which seemed of importance in explaining the spread of bacterial disease it was suggested (1) that the readiness with which an organism initiates infection may depend on its state of growth activity. Especially in the case of the respiratory tract where there exists a highly organized eliminative mechanism which rapidly disposes of foreign particles and bacteria (2) it seemed probable that organisms which on entry were in an inactive growth phase might be eliminated before colonization could take place, whereas bacteria in a highly active vegetative state might under the same conditions take advantage of a portal of entry and produce disease or set up a carrier state. It was pointed out further that such variations in the invasive property that are dependent merely on vegetative activity must be clearly distinguished from specific alterations in virulence whereby a special strain has become adapted to growth in a particular species of animal.

The present experiments were done in the attempt to investigate the above hypothesis. It was not feasible to make tests under conditions which exactly paralleled those existing in the upper air passages of man, and the procedure finally adopted consisted of testing the power of bacteria in various stages of growth activity to initiate infection in the peritoneal cavity of the mouse.

Streptococcus.

It may be recalled that the growth of bacteria in artificial media can be divided into (1) the latent period (time which elapses between seeding and beginning of rapid growth), (2) the logarithmic period

(during which multiplication occurs at a maximum and constant rate), (3) the stationary period (during which growth is very slow or stationary), and (4) the period of decline (during which the organisms are dying). Curves which show graphically the course of events may be plotted in terms of the number of viable bacteria in the culture at various intervals after seeding. For detailed discussion of the biological aspects of bacterial growth phases, and for many illustrative curves the reader is referred to the papers of Penfold (3), Graham-Smith (4), and Chesney (5).

Chart 1 shows the growth cycle of Streptococcus 205 as indicated by counting samples from a broth culture at various time intervals

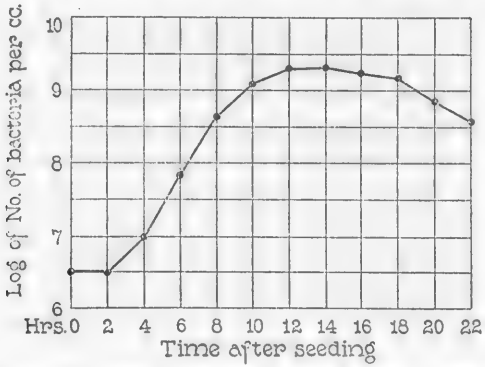


CHART 1. Composite growth curve of streptococcus.

after seeding. The details of such curves vary with many factors such as size of inoculum, age of parent culture, medium, etc., but on the whole the chart may be taken as representative of the cultures used in the present work.

No attempt was made accurately to correlate pathogenicity with rate of multiplication. It was desired merely to compare the pathogenicity of bacteria from the same culture in the stage of active growth and in the period of decline.

Technique.

Flasks of broth were seeded from stock blood agar slants of hemolytic streptococcus or from old broth cultures. After incubation for a specified length of time

a portion of the culture was removed, centrifuged, and resuspended in broth. This material was injected intraperitoneally into series of mice, in amounts which varied although total volume was constant. At the time of inoculation a count was made so that regardless of stage of culture the number of viable bacteria introduced was known. The mice were studied by peritoneal puncture at various periods after injection, time of death was noted, and specificity of death was checked by autopsy and cultures.

Experiment 1.—A 200 cc. flask of meat infusion broth (pH 7.4) was seeded from a stock blood agar slant of Strain 205. After incubation for 4 days a portion of the culture was removed, centrifuged, resuspended in broth, and injected in decreasing amounts intraperitoneally into a series of mice. At the same time 1 cc. of the culture was put into a fresh flask of 200 cc. of broth. After 5 hours incubation this actively growing culture was tested in a similar manner.

Mouse No.	No. of viable bacteria injected.	Peritoneal smear (24 hrs.).		Result.
		White blood corpuscles per field.	Streptococci.	
	Titration of 4 day culture.			
1	5,000,000	300	0	S.
2; 3; 4; 5; 6; 7; 8	2,400,000; 600,000; 150,000; 37,500; 9,200; 1,400; 300	2–300	0	"
	Titration of 5 hr. culture.			
1; 2; 3; 4	25,000,000; 12,000,000; 3,000,000; 740,000	Few.	∞*	D.
5	185,000	"	Few.	"
6	46,000	Many.	"	S.
7; 8	11,500; 2,100	"	0	"

* ∞ = innumerable.

Comment.—It is seen that the maximum dose of 4 day culture which failed to infect was 5,000,000, whereas the maximum dose of 5 hour culture which failed to infect was 46,000, or roughly 1/100 of the former.

Experiment 2.—A flask of 200 cc. of meat infusion broth (pH 7.4) was seeded from a stock blood agar slant of Streptococcus R. After 5, 24, and 72 hours portions of culture were removed and titrated as in Experiment 1.

Mouse No.	No. of viable bacteria injected.	24 hr. smear.		Result.
		White blood corpuscles per field.	Streptococci.	
	5 hr. culture.			
1	28,000,000	20	∞	D.
2	7,000,000	10	∞	"
3	1,750,000	25	∞	"
4; 5; 6	437,000; 109,000; 27,000	1–200	0	S.
	24 hr. culture.			
1; 2; 3	416,000,000; 104,000,000; 26,000,000	Few.	∞	D.
4	6,500,000	50	Few.	S.
5; 6	1,625,000; 406,250	1–200	0	"
	3 day culture.			
1	102,000,000	Few.	∞	D.
2; 3; 4; 5; 6	25,500,000; 6,370,000; 1,590,000; 390,000; 97,000	1–200	0	S.

From the 3 day culture used in the preceding experiments 2 cc. were transferred to a fresh flask of 200 cc. of broth. After 5 hours incubation a titration was done as before.

Mouse No.	No. of viable bacteria injected.	24 hr. smear.		Result.
		White blood corpuscles per field.	Streptococci.	
	Second 5 hr. culture.			
1	140,000,000	Few.	∞ *	D.
2; 3; 4; 5	35,000,000; 8,750,000; 2,180,000; 540,000	"	∞	"
6	135,000	About 100.	25	S.
7; 8	33,700; 8,000	100–200	0	"

* ∞ = postmortem.

Comment on Experiment 2.—The minimal lethal doses of the 5, 24, and 72 hour cultures were respectively 1,700,000, 26,000,000, and 102,000,000. Transfer from 3 day culture yielded a 5 hour culture, the minimum lethal dose of which was 540,000. The quantitative relationships are therefore evident and may be tabulated as follows:

	5 hr.	24 hr.	3 day.	5 hr.
M.L.D.	1,700,000	26,000,000	102,000,000	540,000
Ratio (approximate).	4	50	200	1

It is seen that the pathogenicity of the bacteria was much lower in the stage of decline than in the stage of active growth.

Theoretically the technique of the experiment is quite simple; practically it was found to be very difficult. In fact so many possible sources of error may be thought of that they must be considered in some detail.

1. The Cultures.—Two strains of hemolytic streptococcus, No. 205 and R, were used. Both were derived from cases of acute tonsillitis. They were shown to be colony-pure by plating. Strains derived from single cells were not employed. The protocols, however, show nothing which would suggest that error resulted from absence of pure line strains.

2. Bacterial Counts.—In comparing the action of bacteria in different stages of growth it is obvious that one must reckon with the number of viable bacteria and not merely with gross amounts of culture. Extremely careful counts were therefore made in each case just before inoculation. The culture was carried through a series of eleven dilutions. The tubes were thoroughly shaken at each stage and a fresh pipette was used. Plates (blood agar) were finally poured from the last three dilutions which represented respectively 1/64,000, 1/256,000, and 1/1,024,000 cc. Unless the number of bacteria per cc. of culture as calculated from the colony count of each of the three plates agreed the experiment was not considered valid.

It must be remembered also that plate counts indicate number of colonies and not single bacteria, especially when one deals with such an organism as streptococcus. The preparations were therefore checked by stained smears. Clumping was always more marked in the older cultures and hence any error would be in the direction of minimizing rather than exaggerating the number of organisms in the old culture as compared with the young, and therefore would tend to minimize rather than to exaggerate differences in virulence.

3. Influence of Dead Bacteria in Old Culture.—An examination of the diagram of growth curve of streptococcus shows that in a 3 day culture there must be a great number of dead bacterial bodies—in fact they greatly outnumber the viable organisms. While these dead bacteria may act in a deleterious way, the opposite possibility comes to mind; namely, that they might stimulate leucocytes or protective substances and that hence the apparent decrease in pathogenicity of the old culture might be spurious. This matter was checked by the following experiments.

Experiment 3.—Two sets of six mice received decreasing amounts intraperitoneally of a 5 hour culture of Streptococcus R in 0.5 cc. of broth. Immediately after inoculation Set A each received in 0.5 cc. broth the growth from 1 cc. of 3 day culture which had been killed by heating at 70°C. for 1 minute and Set B each received 0.5 cc. of broth.

Set A.				Set B.			
No.	5 hr. culture.	Killed 3 day culture.	Result.	No.	5 hr. culture.	Broth.	Result.
	cc.	*cc.*			*cc.*	*cc.*	
1	1/2	1/2	D.	1	1/2	1/2	D.
2	1/8	1/2	"	2	1/8	1/2	"
3	1/32	1/2	"	3	1/32	1/2	"
4	1/128	1/2	"	4	1/128	1/2	"
5	1/500	1/2	"				
6	1/2,000	1/2	"	5	1/500	1/2	S.
				6	1/2,000	1/2	"

This experiment shows that the dead bacteria have a deleterious effect which enhances the activity of the living organisms. If one refers then to Experiments 1 and 2 it is evident that the true decrease in pathogenicity of the old cultures was even greater than that which was actually demonstrable.

4. Technique of Comparative Experiments.—This was kept as nearly uniform as possible. The cultures were centrifuged at uniform speed for 10 minutes, and broth from the same lot warmed to incubator temperature was used.

5. Progressive Deterioration of Virulence.—The possibility that differences in pathogenicity of young and old cultures might simply be part of a progressive deterioration of virulence had to be considered. This eventuality was controlled by a second test of a young culture derived from the old cultures previously examined (see Experiment 2).

6. Variations in Susceptibility of Breeds of Mice.—It is well known that white mice of various breeds vary greatly in their susceptibility to certain infections. This has been clearly shown by Webster (6) and others. In the present experiments all the animals came from the same breeder and to a considerable extent from the same hutch.

7. Variations in Susceptibility of Individuals.—Even in mice of the same breed there are distinct differences in susceptibility of individuals to a given infection. A large number of preliminary experiments showed, however, that such individual variations were never greater than sixteen-fold.

The preceding experiments demonstrated that there were distinct differences in the capacity to initiate infection of young and old cultures. As pointed out, no attempt was made accurately to correlate such a phenomenon with the exact rate of multiplication of the bacteria. It seemed of interest, however, to discover whether the diminution in pathogenicity took place abruptly when the culture reached the stationary period or whether there was a more gradual alteration.

Experiment 3 A.—Streptococcus B (beta-hemolytic). Same general technique as in preceding experiments. The pathogenicity of the culture was titrated in mice at intervals of 3, 8, 19, and 26 hours after seeding. Counts were made at the same time so that a growth curve could be constructed. The results are summarized in the following protocol.

Summary of Experiment 3 A.

Age of culture.	Count per cc.	Minimum lethal dose.
hrs.		
3	13,000,000	3,000,000
8	52,000,000	800,000
19	90,000,000	7,000,000
26	100,000,000	16,000,000

Comment.—The first two titrations were made during the stage of active growth; the last two at a time when the culture had entered the stationary period. While the trend may indicate a slight decrease in pathogenicity, the differences were not striking, and it is clear that there was no abrupt alteration.

Experiment 3 B.—Streptococcus Sc (beta-hemolytic). Same procedure as in Experiment 3 A. Titrations were done 8, 10, and 13 hours after seeding. The results are summarized in the following protocol.

Summary of Experiment 3 B.

Age of culture.	Count per cc.	Minimum lethal dose.
hrs.		
8	70,000,000	300,000
10	120,000,000	1,800,000
13	120,000,000	450,000

Comment.—Result similar to that of Experiment 3 A. It is seen that virulence was unaltered after the culture entered the stationary period.

The two preceding experiments showed that the alterations in virulence previously described did not parallel changes in rate of multiplication. It appeared that the culture must enter the stage of decline before material alteration was apparent. In the next experiment several titrations were made during this stage.

Experiment 3 C.—Streptococcus B (beta-hemolytic). The organism was passed through a mouse and recovered in pure culture. This was used for seeding the flask from which the titrations were made. Titrations at 5, 26, 29, and 48 hours after seeding. The results are shown in the protocol.

Summary of Experiment 3 C.

Age of culture.	Count per cc.	Minimum lethal dose.	Ratio.
hrs.			
5	100,000,000	23,000	1
26	300,000,000	2,750,000	120
29	250,000,000	2,250,000	98
48	90,000,000	5,620,000	244
5	8,000,000	8,000	

Comment.—A reference to the growth curve (Chart 1) shows the 26 hour titration was made within a few hours after the culture went into the stage of decline. A marked depreciation of pathogenicity was already present which did not vary a great deal during the ensuing 24 hours.

It appears, then, that with the stage of decline there comes not a gradual, but an abrupt falling off in virulence, although a high and constant level is maintained during the phase of active growth and in the stationary period.

Pneumococcus.

Experiments with pneumococcus were planned, although it was recognized that clean-cut results might not be forthcoming, owing to the extreme susceptibility of mice to pneumococcus infection. We desire to find out, however, whether in the case of an old culture latent period could be demonstrated in the animal body.

Experiment 4.—A strain of Pneumococcus Type I highly virulent for mice was used. 0.01 cc. of culture from a stock blood broth tube was planted in a flask of 200 cc. of meat infusion broth pH 7.8 to which 10 cc. of sterile ascitic fluid had been added. Titrations in series of mice were done with portions of this culture during the active stage (6 hours) and during the period of decline (31 and 54 hours). The same technique was followed as with the streptococcus experiments, and special care was taken in the plating and counting of colonies. Peritoneal punctures were done at 18 hours, and the number of leucocytes and of pneumococci per oil immersion field was noted. Time of death was recorded and autopsies and post-mortem cultures were made. In the protocols Sp. indicates specific death as evidenced by overwhelming pneumococcus infection of peritoneum and blood stream.

Mouse No.	Amount of culture injected.	No. of viable pneumococci injected.	18 hr. peritoneal puncture.		Result.	Post-mortem.
			White blood corpuscles per field.	Pneumococci.		
	Titration of 6 hr. culture of Pneumococcus Type I.					
	cc.					
1	1/1,000	11,000	25	∞	D. 18 hrs.	Sp.
2	1/10,000	1,100	25	4–10	" 30 "	"
3	1/100,000	110	10	4–20	" 42 "	"
4	1/1 million	11*	100	2–4	" 42 "	"
5	1/10 "	1*	100	Less than 2.	" 42 "	"
6	1/100 "	0	100	0	S.	
7	1/1 billion	0	100	0	"	
8	1/10 "	0	100	0	"	

* Actual plate count. Other doses calculated from these.

Comment.—It is seen that with the active (1 hour) culture all the mice, even those receiving as few as 11 and 1 (pair) pneumococci, died a specific death within 42 hours.

No.	Amount of culture injected.	No. of viable pneumococci injected.	18 hr. peritoneal puncture.		Result.	Postmortem.
			White blood corpuscles per field.	Pneumococci.		
	Titration of same culture after incubation for 31 hrs.					
	cc.					
1	1/10	860,000	25	+++*	D. 41 hrs.	Sp.
2	1/100	86,000	100	++	" 41 "	"
3	1/1,000	8,600	Few.	50–100	" 26 "	"
4	1/10,000	860†	50	20–40	" 41 "	"
5	1/100,000	86	100	2	" 65 "	Probably not specific. Peritoneum shows many coarse Gram-positive rods. Heart's blood culture negative for pneumococci.
6	1/1 million	8†	100	0	S.	
7	1/10 "	0	100	0	"	
8	1/100 "	0	100	0	"	

* ++++, +++, ++, and + indicate decreasing numbers of bacteria on a scale of ∞ to about 100 per field.

† Actual plate count. Other doses calculated from these.

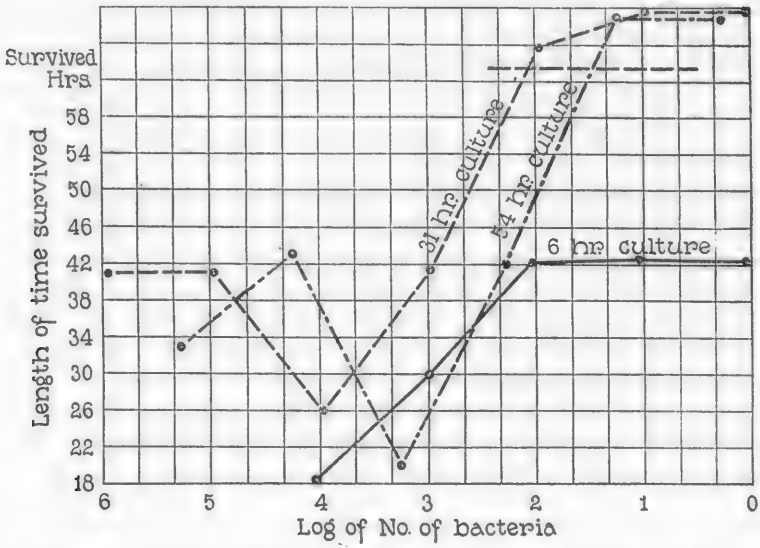
Comment.—The animals not only survived longer than in the case of the 6 hour culture but the smaller doses (8, 86) failed to kill.

No.	Amount of culture injected.	No. of viable pneumococci injected.	18 hr. peritoneal puncture.		Result.	Postmortem.
			White blood corpuscles per field.	Pneumococci.		
	Titration of same culture after incubation for 54 hrs.*					
	cc.					
1	10	2,300,000	Few.	∞	D. 20 hrs.	Sp.
2	1	230,000	50	+	" 33 "	"
3	1/10	23,000	25	50	" 43 "	"
4	1/100	2,300†	100	25–50	" 20 "	"
5	1/1,000	230	100–200	0	" 42 "	"
6	1/10,000	23†	100–200	0	S.	
7	1/100,000	2†	100–200	0	"	
8	1/1 million	0	100–200	0	"	

* In this case the culture was centrifuged and the bacteria were resuspended in broth before dilutions were made.

† Actual plate count. Other doses calculated from these.

Comment.—Essentially the same findings as with 31 hour culture. Mouse 4 was exceptional in so far as it died early, with relatively few pneumococci in the peritoneum. However, culture from the heart's blood was positive and the death must be regarded as specific in a specially susceptible animal.



Each dot represents one animal. The dots above the horizontal broken line represent survivors.

CHART 2. Experiment 4.

The results of Experiment 4 are charted graphically in Chart 2 with logs of number of bacteria injected as abscissæ and number of hours until death as ordinates.

In order to rule out any progressive loss of specific virulence on the part of the culture a portion of that used in the previous experiment was transferred to a fresh flask of broth and the titrations were repeated with the second culture after 6 and 29 hours incubation (Experiment 5).

Experiment 5.

No.	Amount of culture injected.	No. of viable pneumococci injected.	18 hr. peritoneal puncture.		Result.	Post-mortem.
			White blood corpuscles per field.	Pneumococci.		
	Titration of second culture 6 hrs. after inoculation.					
	cc.					
1	1/10	1,500,000	Few.	∞	D. 17 hrs.	Sp.
2	1/100	150,000	50	∞	" 40 "	"
3	1/1,000	15,000	25	++	" 40 "	"
4	1/10,000	1,500	25	++	" 31 "	"
5	1/100,000	150*	Few.	+++	" 30 "	"
6	1/ 1 million	16*	25	10	" 47 "	"
7	1/ 10 "	2*	50	+	" 32 "	"
8	1/100 "	0	50	0	S.	

* Actual plate count. Other doses calculated from these.

Comment.—The 6 hour culture is more pathogenic as measured by time of death of the animals and minimum lethal dose than the 2 day culture from which it was derived.

No.	Amount of culture injected.	No. of viable pneumococci injected.	18 hr. peritoneal puncture.		Result.	Postmortem.
			White blood corpuscles per field.	Pneumococci.		
	Titration of second culture after 29 hrs. incubation.					
	cc.					
1	1	60,000,000	Few.	+	D. 19½ hrs.	Sp.
2	1/10	6,000,000	0	∞	" 22 "	"
3	1/100	600,000				Non-specific. D. 18 hrs. Cannibalism.
4	1/1,000	60,000	25–50	++++	" 24 "	Sp.
5	1/10,000	6,000	Few.	6–10	" 42 "	Non-specific death. No pneumococci in peritoneum or heart's blood.
6	1/100,000	600*	25	2	" 50 "	" "
7	1/1 million	60*				D. 18 hrs. Non-specific. Cannibalism.
8	1/10 "	7*	Few.	0	S.	

* Actual plate count. Other doses calculated from these.

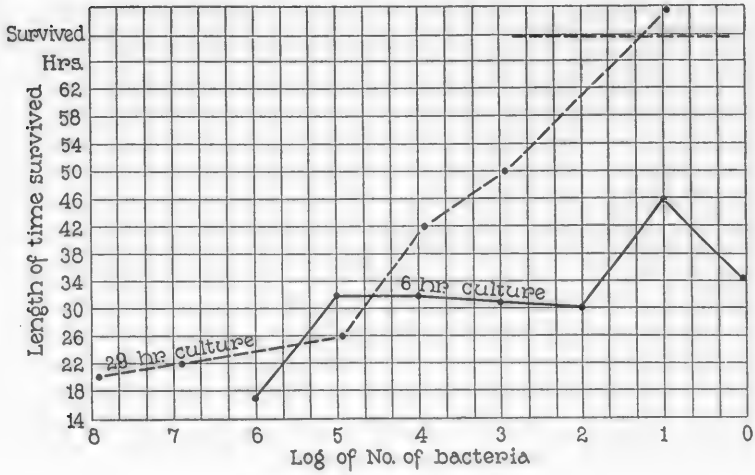


CHART 3. Experiment 5.

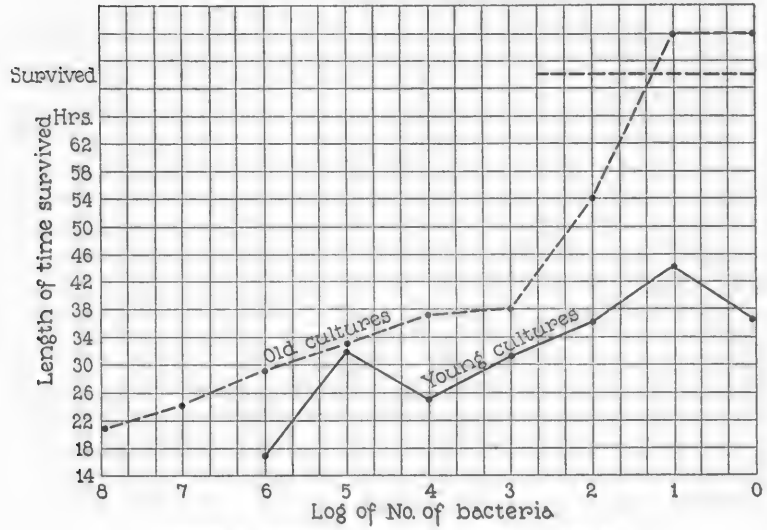


CHART 4. Composite graph of Experiments 4 and 5.

Comment.—Result similar to previous experiments. The non-specific death of Nos. 5, 6, and 7 was unfortunate as these animals might have survived the pneumococcus infection for a greater length of time. The experiment is shown graphically in Chart 3.

Discussion of Experiments 4 and 5.—In Chart 4 the combined results of the two experiments are charted graphically. The solid line shows the average of two titrations of 6 hour cultures, the broken line the average of three titrations of culture in the period of decline. Several facts are brought out: (1) Of the mice which received equal numbers of bacteria from active and inactive stages of cultures, those injected with the highly vegetative organisms uniformly died after a shorter interval. This interval averages (with doses of 100 bacteria or over) about 10 hours. Evidently there occurs with the old culture a period of lag which is quite analogous to that seen in the test-tube. (2) Doses of old culture smaller than 100 bacteria were uniformly survived (five animals), whereas even one pneumococcus from the 6 hour cultures invariably killed (four animals). It is clear then that even with an animal as highly susceptible to pneumococcus as the mouse a definitely lower degree of pathogenicity can be shown in the case of old cultures than in the case of those in the logarithmic phase of growth.

CONCLUSIONS.

The experiments which have been reported show that distinct differences exist between relatively young cultures of bacteria and the same strains during the period of decline as regards invasive power and pathogenicity, and that these differences must be distinguished clearly from specific alterations in virulence such as those produced by animal passage. The exact interpretation of these observations is not, however, perfectly clear. We were inclined to believe that simple alterations in vegetative activity might account for the differences which have been described, but to what extent the results have been due to injury to the bacteria by products of culture growth it is impossible to say, and further work will be necessary to settle this point.

At any rate the experiments seem to bear definitely on the problem of infection in as far as they show that purely temporary modifications

of growth activity whether or not brought about by specific injury lead to changes in invasiveness which are quite analogous to the test-tube phenomenon of lag.

It has previously been shown that there exists in the upper air passages a mechanism by means of which foreign particles and bacteria can be eliminated within a few hours. It seems highly likely on the basis of the present work that bacteria entering in an inactive growth phase—for example dried in dust or perhaps from a chronic carrier—may be disposed of before activity can be resumed, whereas organisms introduced in the stage of active growth—as from a case of acute disease—may be able to take advantage of a portal of entry. It is further possible that these experiments may have some bearing on the genesis of epidemics, especially as regards the preepidemic phase, and these matters will be discussed at another time.

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